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THE EFFECT OF GIBBERELLIC ACID ON GROWTH AND INDOLE METABOLISM OF DWARF PEA PLANTS

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THE EFFECT OF GIBBERELLIC ACID ON GROWTH AND INDOLE METABOLISM OF DWARF PEA PLANTS

by

ZAHEED HUSAIN

A dissertation submitted to the Graduate Faculty in
Biology in partial fulfillment of the requirements for
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May 20, 1986
date

Jack G Valdovinos
Chairman of Examining Committee
Jack G. Valdovinos, Lehman College

August 5, 1986
date

Executive Officer
Peter C. Chabora

J Golubow
Julius Golubow, Lehman College

Neil G. Grant
Neil G. Grant, William Paterson

Thomas E. Jensen
Thomas E. Jensen, Lehman College

Roy E McGowan
Roy McGowan, Brooklyn College

Supervisory Committee

The City University of New York

Abstract

"THE EFFECT OF GIBBERELLIC ACID ON GROWTH AND INDOLE METABOLISM OF DWARF PEA PLANTS

by

Zaheed Husain

Advisor: Professor Jack G. Valdovinos

A study was conducted to describe the pathway of biosynthesis of indole-3-acetic acid (IAA) from tryptophan (TPP) and determine the effect of gibberellic acid (GA_3) on this system. Treatment of dwarf peas (Pisum sativum L. var Little Marvel) with 0.8 ug GA_3/plant resulted in increase in plant height along with increased auxin level. A cell-free preparation of pea shoot tissue was able to convert D,L-tryptophan-3-^{14}C into different indole metabolites. The acidic and neutral fractions obtained after TPP incubation were subjected to thin-layer chromatography. In the neutral fraction, two peaks of radioactivity were found and these matched the Rfs for indole-acetaldehyde (IAAld) and indole-3-ethanol (IEt). One major peak of radioactivity was observed in the radiochromatograms of the acidic fraction and it corresponded with authentic IAA. The IEt observed as a product of tryptophan metabolism was further characterized by TLC in different solvent systems and by color reaction with the Ehrlich and perchloric Salkowski reagents.

The enzymes involved in the conversion of TPP to IAA involved, in the first step, a transaminase (tryptophan aminotransferase, EC 2·6·1) reaction. The aminotransferase was purified about 82-fold by acetone precipitation and Sephadex G-200 filtration. It had a pH optima of 8.5 and a temperature optima of 40°C. With α-ketoglutarate a co-substrate, the enzyme transaminated aromatic as well as alphatic amino acids including D,L-tryptophan, D,L-alanine and D,L leucine. D-TPP was found to be more effective than L-TPP as a substrate. Among the keto acids tested, pyruvic and oxalacetic acid were equally effective as substrates. The K_m value for the transaminase found to be 2.5×10^{-4} M D,L-TPP. Stoichiometric yields of IPyA (1.18 u moles) and glutamic acid (1.20 u moles) prove that IPyA formation is by the transamination reaction. GA_3 treatment to dwarf pea plants results in increase in the specific activity of the enzyme over the observation period. In the second step of TPP conversion, IPyA is decarboxylated by an enzyme to IAAld. In plants treated with GA_3, the enzyme activity was significantly higher three days after treatment but remained unaffected at all other stages when observations were made. The final step enzyme is a dehydrogenase that can convert IAAld to IAA in the presence of MAD as a co-factor. The dehydrogenase enayme was partially purified and three peaks of activity were resolved after gel filtration (M.W. 9,200, 22,500 and 40,000 daltons). The enzyme had a pH optima of 5.5. Treatment of plants with GA_3 caused increased activity of the enzyme, with the maximum increase occurring one day after treatment.

Dwarf pea tissue was also found to convert IAAld to IEt by the activity of a reductase enzyme and it was purified about 75-fold. One major protein peak was obtained after Sephadex filtration (M.W. 40,000 daltons). The enzyme required either NADPH or NADH as a co-factor and had a K_m of 208 uM of

IAAld. It had a major pH optima at 7.0 and a secondary optima at 5.5. The activity of the enzyme in GA-treated plants was higher than controls up to the third day, declined and then increased again around the sixth day following treatment. The presence of IAA in the enzyme assay, drastically reduced the conversion of IAAld to IEt. This result contradicts the suggestion that IEt is a storage product diverting away from excessive IAA production. Moreover, incubation of IEt in crude enzyme brei did not yield IAAld in a reversible reaction. It is suggested that IEt itself has a more important regulatory role in plant growth and three different bioassays showed it to be biologically active.

ACKNOWLEDGEMENTS

Sincerest appreciation is extended first and foremost to Prof. Jack G. Valdovinos, who provided the intellectual guidance for the research and the moral support to help me attain this goal. Thanks are also extended to Professor Thomas E. Jensen (who helped me in so many different ways) and Dr. Jules Golubow for suggestions during the course of the experiments.

This work would not have been a reality without the support of my family. I wish to express my deepest gratitude and appreciation to my wife Laila who, not only stood by my side through the most difficult times, but also provided invaluable assistance. My son Aiyaz deserves special thanks and appreciation for all his help and patience. I also appreciate the help provided by Mr. Kazi Belal in preparing the photographs and slides.

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Finally, I would like to dedicate this work to the memory of my father who wanted, more than anyone, to see it completed.

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Introduction

There has been considerable interest in the chemistry and physiology of the gibberellins over the years and the subject has also been extensively reviewed (Paleg, 1965; Key, 1969; Lang, 1970; Yomo and Varner, 1971; Jones, 1973; Hedden, et al. 1978).

Gibberellins (GAs) are synthesized in the apical region of the shoot (Jones and Phillips, 1966, in sunflower) and in the roots (Sebanek, 1966, in pea). They move freely within plants in a non-polar manner both acropetally from the cotyledons and basipetally from the shoot apex of pea plants (Moore, 1967). McComb (1964) using intact dwarf pea seedlings, found movement of labelled GA_3 from the mature leaves to young leaves.

Exogenous applications of GAs have been found to produce a wide variety of effects in plants. Thus, GA treatment promotes stem elongation and flowering in rosette biennial plants, promotes stem and fruit growth, reverses some types of genetic dwarfism, releases vegetative buds from dormancy and causes synthesis of α-amylase in aleurone layers in cereal grains. On the other hand, GA_3 inhibits red light-promoted growth, sucrose uptake (Goren and Galston, 1967), decrease protein content and peroxidase activity (Galston, 1977) but has no effect on the movement of photosynthetic assimilates in peas (Harvey, 1977).

Most of the positive effects of GAs on stem elongation are attributed to the effects on cell elongation, cell division or both.

The evidence that GAs affect growth by stimulating cell elongation comes from work with gamma plantlets of wheat, epicotyl sections of lentil and internode sections of *Avena* (Haber and Luippold, 1960; Nitsan and Lang, 1966; Rose and Adamson, 1969). By inhibiting cell division with gamma radiation, Haber and Luippold (1960) and Rose and Adamson (1969) were able to demonstrate that the effect of GA on growth was by cell elongation only. Kefford and Rijven (1960) also showed that embryos isolated from irradiated wheat seeds would still respond to GA by cell elongation. Growth in wheat coleoptiles have been separated into three distinct phases, the earliest of which is by cell elongation alone. Gibberellic acid has been shown to promote the elongation phase of growth mostly (Wright, 1961; 1966). Kaufman _et al_. (1969) and Jones and Kaufman (1971) observed that GA_3 suppresses cell division in the intercalary meristem of _Avena_ internodes but stimulates growth in this tissue by cell elongation. Epicotyl sections of lentils were also observed to respond to GA_3 application by cell elongation (Nitsan and Lang, 1966).

The effect of GAs in stimulating stem growth of rosette plants involves both cell elongation and cell division. Sachs _et al_. (1959) reported that the preliminary effect of GA_3 in _Hyoscyamus_ and _Samolus_ was promotion of cell division but not elongation. Stimulation of cell division following GA application has also been reported by various workers (Bradley and Crane, 1957; Wareing _et al_., 1964; Shininger, 1971).

Lockhart (1956) suggested that radiation-induced growth inhibition of pea was brought about by changes in cell turgor mediated by changes in wall plasticity or by changes in the osmotic concentration of the cell. Although no difference was found in the osmotic concentrations of cell sap obtained from pea plants grown in darkness or in light, estimation of wall extensibility indicated that light markedly reduced both elasticity and plasticity of internode cells. It was also shown that GA_3 could overcome the reduction of cell wall plasticity resulting from irradiation.

Cleland _et al_. (1968) reported that although GA promoted elongation of cucumber hypocotyls, it had no effect on wall plasticity of cucumber hypocotyl sections and suggested that GA-stimulated growth of cucumber hypocotyl sections resulted from a change in the osmotic concentration of the cell.

Biochemical aspects

The number of reports showing effects of GAs on DNA, RNA and protein metabolism in growing tissues are quite significant (Key, 1969; Glasziou, 1969; Jacobsen, 1977).

Nitsan and Lang (1966) reported that in lettuce hypocotyl and lentil epicotyl tissue GA-stimulated elongation was dependent on synthesis of DNA and it could be inhibited by 5-fluordeoxyuridine (FuDR). Broughton (1968, 1969) and Holm _et al_. (1970) demonstrated a dependence of cell elongation on DNA synthesis in pea and soybean respectively. However, treatment of soybean epicotyls with FuDR did not cause inhibition of GA-induced growth although it did inhibit DNA synthesis (Holm _et al_., 1970). Also, the marked elongation growth of dwarf pea internodes by GA_3 cannot be blocked by FuDR (Broughton, 1968; 1969). Such findings have also been reported by other workers (Haber _et al_., 1969; Rose and Adamson, 1969).

Johri and Varner (1968) first reported that GA could enhance the DNA dependent RNA synthesis in isolated pea nuclei. GA also promoted

RNA synthesis in isolated pea nuclei. GA also promoted RNA synthesis in sugar beet nuclei (Duda and Cherry, 1971). Examination of isolated chromatin showed that while GA can affect both template availability and polymerase activity, the response seems to differ from tissue to tissue (McComb _et al_., 1970; Duda and Cherry, 1971).

Regulation of in vivo RNA metabolism

GA treatment has increased the RNA content of a number of plant species including pea seedlings, clover seedlings, lentil epicotyls, barley leaf segments and senescing leaf tissue (Broughton, 1969; Key, 1969; Poulson and Beevers, 1970). Application of gibberellic acid has also been shown to increase incorporation of precursors into all major species of RNA in pea epicotyls (Nakamura _et al_., 1977), corn embryos (Wielgat et al., 1974), protoplasts of corn seedlings (Wasilewska and Kleczkowski, 1974) and barley leaves (Poulson and Beevers, 1970).

In barley aleurone tissue, it is clear that synthesis of some RNA species is necessary for amylase production. There seems to be sufficient evidence to support that this is mRNA(Zwar and Jacobsen, 1972).

Gibberellin effect on auxin levels

A very remarkable effect of GA is its ability to overcome dwarfism caused by mutation of a single gene. Application of gibberellic acid to such dwarf plants (e.g. _Pisum sativum_, _Vicia faba_ and _Phaseolus multiflorus_) causes increase in plant height so that treated plants become as tall as normal plants. Increased concentration of GA increases the growth response of these plants. Gibberellin-induced growth has been suggested to be mediated through increased auxin production (Kuraishi and Muir, 1962). The increased auxin level then causes increased growth of treated plants.

In general, a reasonably good correlation is found between relative auxin content and relative growth in the various organs of seed plants, particularly taking into account the marked differences in sensitivity of the different parts of a plant to auxin. Thus, while relative growth rate increased with increasing concentrations of auxin, the amount of diffusible auxin was observed to be higher from the apex to the base of the plant (Scott and Briggs, 1960). At the same time, it has been shown that IAA oxidase activity is also negatively correlated with auxin levels so that the oxidizing activity is low in regions of high auxin content, (Galston, 1977).

Moore (1969) also reported a good correlation between the capacity for net auxin biosynthesis from tryptophan and the endogenous free auxin content of various parts of green pea seedlings. Based on incorporation of 2-^{14}C labelling into ^{14}C-IAA, he showed that all parts of the shoot could convert TPP into IAA with the most active auxin production taking place in the terminal bud of pea plants.

Nitsch (1957) reported that Rhus plants had more extractable auxin in its apex during long days than under short days. Moreover, GA_3-treated long and short day plants had more auxin as compared to controls. In these experiments the auxins were extracted with methanol chromatographed and then tested with the coleoptile bioassays. Nitsch and Nitsch (1959a,b) also obtained similar increases in auxin levels of dwarf kidney beans following GA_3 treatment. Kuraishi and Muir (1963) found that GA_3 treatment caused a forty-fold increase in the auxin level of rosette *Hyoscyamus* plants, using auxin diffusion and *Avena* bioassay techniques.

Application of GA_3 has been found to cause increased auxin levels in dwarf and tall peas (Phillips *et al*., 1959; Kogl and Elema, 1960; Kuraishi and Muir, 1962, 1963; Valdovinos *et al*., 1967), sunflower (Kuraishi and Muir, 1962), *Parthenocissus* tissue culture (Butenko *et al*., 1961), winter wheat vegetative tissue (Michiniewicz, 1962), *Hyoscyamus* and *Centaurea* (Kuraishi and Muir, 1963; Muir and Kuraishi, 1963), parthenocarpic tomato (Sastry and Muir, 1963) and Coleus petioles (Muir and Valdovinos, 1970). Also, *Betula* (Kawase, 1961), azalea, *Rudbeckia* and Perilla (Lebedenko, 1963), rice (Yerygin *et al*., 1961), potato (Reingard and Palladina, 1963), cabbage and corn (Radionova and Runkova, 1963) and celery (Kato and Ito, 1962) have been shown to have increased auxin levels following GA treatment.

In the light of the above results, it is quite pertinent to consider the exact mode of this increased auxin production following GA treatment of plants. Among the different possibilities that exist are the following:

1. GA promotes the destruction of IAA by inhibiting an oxidase enzyme.
2. It promotes the protection of a substance that protects IAA.
3. It increases polar transport of auxin.
4. It increases the biosynthesis of IAA by acting on enzyme(s) involved in the pathway for IAA production.

Effect of GA on IAA oxidation

The level of extractable IAA oxidase and peroxidase were decreased in dwarf pea and corn with GA treatment (Galston and McCune, 1961). Valdovinos and Ernest (1966) reported a decrease in IAA destruction following GA treatment of dwarf pea plants. Kogl and Elema (1960) found lower oxidase activity in crude preparations of tissue extracts of GA-treated peas but, when purified, there was no difference in activity between treated and control plants. Pilet (1961) reported a decrease in oxidative degradation of IAA in Lens root sections treated with GA_3. His results suggest that the greatest inhibition was in older cells and less inhibition occurred as the tip was approached. It was also suggested that although GA_3 may cause an inhibitory effect on oxidative activity in excised tissues, in vivo it has a neutral or stimulatory effect at a concentration of 5×10^{-4}M after 24 hr. A fifty percent decrease in the auxin destruction activity was found upon addition of GA_3 (10^{-3}M) to an extract from crown gall tissue (Witham and Gentile, 1961). The authors however suggest that the GA_3 itself may have broken down at the low pH that was used.

Halevy (1962, 1963) reported decrease in peroxidase and "IAA oxidase" activity caused by GA_3 treatment. Results were obtained in the above experiments from 4 day old seedlings germinated in test solutions. He observed change in the oxidative activity but not in the peroxidative activity following GA treatment.

McCune (1961), and Galston and McCune (1961) reported the change in isozymes capable of oxidizing IAA following GA_3 treatment of dwarf maize.

To summarize the above reports, it can be concluded that while GA has been shown to have no effect on the purified enzyme in vitro

(Watanabe and Stutz, 1960; Kogl and Elema, 1960; Brian and Hemming, 1958), there have been reports of both positive and negative effects of GA on IAA oxidative activity in various plants (Kato and Katsumi, 1958; Housely and Deverall, 1961; Murakami and Hyashi, 1963).

Effect on IAA protector substances

Watanabe and Stutz (1960) observed an increase in inhibitor concentration in some tissues under certain light conditions following GA_3 treatment. Galston (1959) reported an increase in inhibitor content of pea seedlings following GA_3 treatment but no change in the oxidative activity in pea stems over a 100-fold concentration range of GA_3. Kogl and Elema (1960) reported increased amounts of polyhydroxycinnamic acids (ferubic, caffeic and chlorogenic acids) after GA_3 treatment. However, Sagi and Garay (1961) found no effect of GA_3 on either oxidative activity or phenol content in leaves of treated plants. Kuraishi and Muir (1964) also reported the inability to detect any diffusible inhibitor substance from GA treated dwarf pea plants.

Auxin-protector substances have been reported by several workers to be present in plants and these have been found to prevent enzymatic destruction of IAA (Morgan and Hall, 1963; Phillips and Jones, 1965; Stonier and Yoneda, 1967; Yoneda and Stonier, 1967). Stonier and coworkers have reported three high molecular weight substances from shoots of *Pharbitis nil*. These substances, named protector A (MW 200,000), protector I (MW 8,000) and protector II (MW 2,000) were obtained by gel filtration of crude extracts. Stonier (1970) considers that these protectors induce a lag in the oxidation of IAA by HRP by interacting with H_2O_2 and by also keeping Mn in a reduced state. In Pharbitis, these protector substances are present in a diminishing gradient from younger to older tissue (Yoneda and Stonier, 1967). Other substances that have been reported to alter the activity of peroxidase and IAA oxidase include phenols, coumarins, manganese salts and plant acids (Schneider and Wightman, 1974).

Imbert and Wilson (1970) found that scopoletin, which is a naturally occurring coumarin, would inhibit IAA oxidase at higher concentrations but stimulate it at lower concentrations in sweet potato. Sirois and Miller (1972) reported that scopoletin inhibited IAA oxidation in a competitive manner.

Grambow and Langenbeck-Schwich (1983) found that p-monophenolic compounds (e.g. p-coumaric acid) increased the rate of IAA degradation while the 3,4-disubstituted phenols were generally inhibitory. Such results have also been reported by other workers. Gelinas (1972) obtained logarithmic increases in the lag period prior to peroxidase catalyzed oxidation of IAA with linear increments of ferulic acid. Grambow and Langenbeck-Schwich (1983) concluded that for the peroxidase action, monophenols remove H_2O_2 from the enzyme and make the enzyme more accessible for IAA oxidation. On the other hand, diphenols could steadily cause reductive regeneration of IAA from an oxidized state.

It has been long known that monophenols (2,4-diclorophenol) and ortho-diphenols (e.g. cotechol and pyrogallol) inhibit IAA oxidation. Flavonols act in the same way, that is, kaempherol promotes while quercetin inhibits IAA oxidase activity (Moore, 1979). Pea plants contain the flavonols kaempferol and quercetin as the triglucosides and coumaroyl (coumaric acid) triglucosides. When an etiolated pea plant is irradiated with white or red light, interesting correlative effects of red light occur with respect to the flavonols. Red light promotes apical bud growth and inhibits subapical stem elongation. In a reportedly correlative manner, red light stimulates the synthesis of kaempferol in stems and quercetin in leaves.

Since phenolic substances occur widely in plants, they must be assumed to have some importance as modifiers of auxin destruction (Moore, 1979).

Effect on auxin biosynthesis

The most satisfactory explanation for increased auxin levels is the stimulation of some step in the biosynthetic pathway for auxin. Such a suggestion was first put forward in 1958 (Brian and Hemming, 1958). In fact, Hyashi and Murakami (1953) found that etiolated pea epicotyls would elongate in the presence of tryptophan if GA_3 was also present.

Kuraishi and Muir (1964) found that treatment with 10^{-3} M GA_3 caused an increased formation of a water-soluble auxin as determined by collecting the diffusible auxin followed by *Avena* bio-assay. Valdovinos *et al*. (1967) obtained increased auxin levels following GA_3 treatments of pea tissue and concluded (on the basis of TTP-1-^{14}C metabolism) that GA enhances the decarboxylation of either TTP itself of an intermediate with a 3-carbon side chain prior to the aldehyde stage (Valdovinos and Ernest, 1966).

Auxin biosynthetic pathway

Tryptophan has been considered, because of its structural similarities, to be the precursor of IAA. Even though it is ubiquitously present, not many determinations of tryptophan levels have been made in plant parts. However, the reports available show a somewhat common range of its occurrence e.g. 20-40 ug/g fresh weight in barley leaves (Schneider *et al.*, 1972), 20 ug/g fr wt in wheat leaves (Kim and Rohringer, 1969) and 30 ug/g fr wt in bean shoots (Schneider *et al.*, 1972).

A wide variety of plants have been found to convert C^{14}-TTP to C^{14}-IAA. Such conversions have been reported in watermelon slices (Dannenberg and Liverman, 1957), dwarf pea shoots (Kuraishi and Muir, 1964), cabbage shoots (Wightman, 1962), tomato shoots (Gibson *et al.*, 1972), lime fruits (Khalifa, 1967), cucumber hypocotyls (Sherwin and Purves , 1969), maize coleoptiles (Libbert and Silhengst, 1970), bean shoots (Black and Hamilton, 1971), and bean roots (Mitchell and Davis, 1972). Cell-free preparations of mung bean (Wightwan and Cohen, 1968), tobacco (Phelps and Sequiera, 1967), pea seedlings (Moore, 1969) and pine needles (Sandberg, 1981) have also been reported to be able to convert TTP to IAA.

Pathways for IAA formation

Considering that IAA is formed from TPP there seem to be four possible pathways for which evidence is present.

Indolepyruvic acid pathway:

Khalifa (1967) reported that the incubation of young lime fruits with C^{14}-TTP resulted in the formation of a radioactive substance that had the same rf as authentic IPyA. IPyA was also reported to have been formed after feeding primary leaves of wheat with C^{14}-TTP (Kim and Rohringer, 1969). Gibson *et al*. (1972) demonstrated the *in vivo* conversion of C^{14}-TTP to C^{14}-IPyA by the formation of IPyA hydrazone and identification on TLC plate.

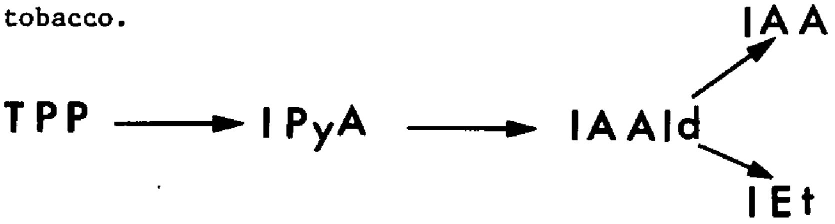
Truelsen (1972) obtained conversion of TTP to IPyA and monitored it by trapping the acid as its borate complex and measuring it at 328 nm. The IPyA formed was also identified by the formation of hydrazone and TLC (Truelsen, 1972).

The first step in the pathway involves a transamination reaction catalyzed by a multispecific aminotransferase. Gamborg (1965) partially purified an aromatic aminotransferase from mung bean seedlings that could transaminate several monoaromatic amino acids also. Forest and Wightman (1972) purified 600-fold an aromatic aminotransferase from bush bean and showed by gel electrophoresis that only a single protein was present. Matheron and Moore (1973) also partially purified from pea shoots an aminotransferase that could transaminate D,L-TTP, D,L-Tyr, D,L-Phenylalanine and some aliphatic amino acids. Truelsen (1973) has demonstrated the presence of such a transaminase in 30 species from 16 families of plants.

The second enzyme for this pathway, IPyA decarboxylase, was purified 234-fold from yeast (Sukanya et al., 1971). Evidence for the presence of this enzyme in crude fractions of tomato shoots was put forward by Gibson *et al*. (1972) but no reports of a purified enzyme has been reported mainly because of the instability of IPyA in aqueous solutions.

In the last step, IAAld is converted to IAA by a NAD-dependent IAAld dehydrogenase (Wightman and Cohen, 1968) or an oxidase (Rajagopal, 1971).

An IAAld oxidase was reported to be present in oat coleoptiles that converted the aldehyde to IAA and has been purified 18-fold by Rajagopal (1971). Such an oxidase has also been reported by Liu _et al_. (1978) in tobacco.



1. TPP aminotransferase
2, IPyA decarboxylase
3. IAAld dehydrogenase or oxidase
4. IAAld reductase

An enzyme forming IAAld to indole-3-ethanol (IEt) has been reported by several workers (Brown and Purves, 1976; Bower _et al_., 1976; Schneider and Wightman, 1974, Liu _et al_., 1978). Rajagopal (1967) reported that of 33 plant species representing 17 lower and higher plant families tested, all could metabilize IAAld to IEt _in vivo_.

The Tryptamine Pathway:

In many species of plants, C^{14}-TPP has been found to be converted to C^{14}-TNH_2 and thence to C^{14}-IAA (Phelps and Sequiera, 1967; Gibson _et al_., 1972). The tryptamine formed is converted to IAA via IAAld whose involvement in this scheme was shown by Gibson _et al_. (1972) in tomato and barley shoots by radiolabelling. The tryptamine pathway was also indirectly proved by reports which showed that

^{14}C-TNH_2 gave rise to C^{14}-IEt in tomato shoots (Gibson et al., 1972), cucumber hypocotyls (Sherwin and Purves, 1969) and in peas (Magnus, *et al.*, 1973).

The first step in this pathway is catalyzed by the enzyme TPP decarboxylase which has been reported from tobacco buds (Phelps and Sequiera, 1968), cucumber hypocotyls (Sherwin and Purves, 1970), barley and tomato shoots (Gibson, *et al.*, 1972) and peas (Muir and Lantican, 1968).

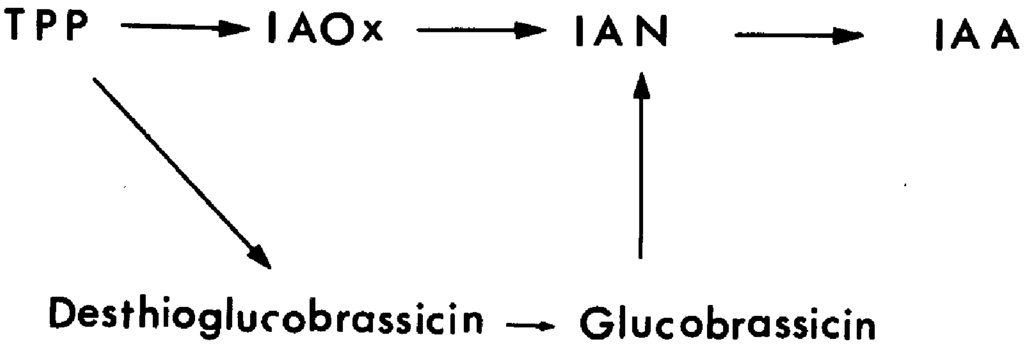
Tryptamine formed during the decarboxylation is converted to AAld by an amine oxidase. Mann (1954, 1961) purified an amine oxidase from pea seedlings that could oxidatively deaminate a number of amines including tryptamine (Hill and Mann, 1964; McGowan and Muir, 1971).

The Indoleacetaldoxime Pathway

This pathway seems to be a characteristic of the Brassicaceae family. Indoleacetaldoxime has been found to promote growth of oat, wheat and maize coleoptiles (Bentley and Housely, 1952; Seeley et al., 1956), pea stem segments (Fawcett, 1964), cabbage, radish, turnips (Michel, 1957), cucumber, sunflower, *Impatiens* and *Salvia* hypocotyl tissues (Ballin, 1962).

Several studies have shown that radioactive TPP can be converted to IAOX, IAN and glucobrassicin in cabbage plants (Kindl, 1968, Kutacek and Kefeli, 1968; Kutacek and Kefeli, 1970). C^{14}-IAN has been observed to be converted to IAA in cabbage, wheat and corn (Wightman, 1962; Evans and Rayle, 1970). Rajagopal and Larsen (1972) reported that IAOX could be converted to IAN in 8 species of plants in large amounts but only in traces in many others. Moreover, almost all the species tested would convert IAOX to IAA and indoleethanol.

The enzyme system responsible for conversion of TPP to IAOX is Kindl's (1968) HRP/dihydroxyfumarate system, but such a system is unlikely to be present in vivo (Schneider and Wightman, 1974). In an alternate system, TPP could be converted to glucobrassicin (Kutacek and Kefeli, 1968) which could be converted to IAN by a myrosinase. Indoleacetaldoxime hydro-lyase, which can convert IAOX to IAN has been reported from banana leaves (Mahadevan, 1963) and Giberella (Shukla and Mahadevan, 1968). IAN can be converted to IAA by nitrilase, an enzyme found to be present in the families Gramineae, Cruciferae and Musaceae (Thimann and Mahadevan, 1964).



Gibberellins and dwarfness

In the field of plant growth, the most important facts which immediately attracted the attention of both geneticists and plant physiologists were the experiments on the effect of gibberellins on genetic dwarfs of pea and maize.

Dwarfism in pea is apparently expressed only in light-grown plants. In etiolated plants, the difference in stem elongation is greatly reduced or is non-existent. Also, etiolated plants show very little response to gibberellin whereas light-grown dwarf cultures of peas and other species usually respond with dramatic stem elongation (Brian and Hemming, 1955).

The effect of light and the involvement of gibberellin in stem elongation have been studied by various workers (Jones and Lang, 1968; Kende and Lang, 1964; Kohler, 1965a,b, 1966, 1970; Kohler and Lang, 1963; Lockhart, 1956, 1959; Lockhart and Gottschall, 1959; Moore, 1967; McComb and McComb, 1970; Broughton and McComb, 1971; Keller and Coulter, 1982).

The dwarfism that is most completely understood at the molecular level is that of the dwarf mutant of corn (*Zea mays*) known as the dwarf-5 (*d_5*) mutant. The *d_5* phenotype is due to a single gene mutation, which drastically reduces the height of plants by reducing the length of the internodes. The dwarfism is expressed from seedling stage to maturity and is observed in etiolated as well as light-grown plants. The dwarf-5 mutant is deficient in endogenous GA, compared to normal genetic strains, and responds by normal growth to exogenous application of GAs and GA precursors as *ent*-kaurene, *ent*-kaurenol and ent-kaurenoic acid. Phinney and Hedden (1976) showed that the d_5 mutation controls the cyclization of copalyl pyropophosphate (B activity of kaurene synthetase) to kaurene. The d_5 mutant produces mainly isokaurene, an intermediate which is not converted to GAs, and only a small amount of ent-kaurene which is a GA precursor. However, normal plants efficiently synthesize ent-kaurene but produce only small amounts of isokaurene.

Genetic dwarfs of several other species appear to be deficient in GAs as compared to their normal or tall counterparts. There is good bioassay evidence for the metabolic steps blocked by the d^1, d^2 and d^3 mutations in maize (Phinney and Spray, 1980) and the dx and dy mutations in rice (Murakami, 1972). Five dwarf cultivors or rice and one dwarf mutant of bean have been reported to be GA-deficient but in 10 dwarf rice cultivors, no differences in GA content, compared to normal plants, could be found (Moore, 1979).

The internode length in peas is controlled by at least 5 major loci, Le, La, Cry, Na and Lm (Reid, et al., 1983). Interaction of these genes can give a wide range of phenotypes from the extremely short nana types (Wellensiek, 1969) through to the elongated slender types (deHaan, 1927). Plants having the homozygous recessive la cry^5 have a high growth rate, irrespective of the allele at the Le locus, and have been termed as "slender" (deHaan, 1927; Rasmussen, 1927). When either or both the dominant alleles are present at La and Cry, the growth rate is decreased, and the effect of the Le locus is observable; plants homozygous for le are "dwarf," and plants with Le are "tall."

The biochemical basis for the difference between tall (genotype Le) and dwarf (type le) cultivars of peas has been the subject of a long standing debate and considerable speculation (e.g. Brian, 1959; Radley, 1958; Jones and Lang, 1968; McComb and McComb, 1970; Chailakhyan, 1979). Brian (1957) suggested that gibberellin synthesis was controlled at the Le locus, and that gibberellin reverses inhibition by La and Cry. Slender plants would therefore elongate in the absence of the hypothetical growth inhibition, while dwarf and tall cultivars, in which growth is inhibited by La and/or Cry, may differ in endogenous gibberellin content.

In a number of studies, a higher amount of endogenous gibberellin has been found in the tall types as compared to dwarf types. This has been shown in maize (Phinney, 1961), bean (Proano and Green, 1968; Gotoh, 1970), sweet pea (Morgan, 1963), tomato (Lockard *et al.*, 1970) and potato (Risch, 1976). On the other hand, numerous reports suggested no direct relationship between endogenous gibberellin level and height of pea plants (Radley, 1958; Galston, 1961; Harada et al., 1968; Suge and Murakami, 1968; Jones and Lang, 1968; Chailakhyan, 1979). Thus, Radley (1958) found no differences in extractable amounts of gibberellin between the tall cultivar "Improved Pilot" and the dwarf "Meteor." Kende and Lang (1964) and Jones and Lang (1968) reported no qualitative or quantitative differences for GA_1- and GA_5-like fractions to be evident between dwarf and normal pea, regardless of growth in red light or darkness.

In a recent work, Keller and Coulter (1982) reexamined the relative levels of GAs in the epicotyls of tall (cv. Alaska) and dwarf (cv. Progress No. 9) seedlings by packed-column gas chromatography (GC) and probe mass-spectrometry (MS). These authors tentatively identified gibberellin A, and GA_5 in both extracts and concluded that GA levels were higher in dwarf than in tall seedlings from GC-quantitation.

Potts et al. (1982) showed that a highly active polar GA-like substance was present in the apical stem tissue of tall (Le) peas but not to any appreciable extent in dwarf (le) peas. Later, this polar GA-like substance was identified as GA_1. Ingram et al. (1983) also observed that application of GA_1 could mask the Le/le gene difference. However, while Le plants responded equally to GA_{20} and GA_1, le plants responded very slightly to GA_{20}.

When plants were treated with GA_{20}, Le plants metabolized it to GA_1, GA_8 and GA_{29} whereas le plants produced GA_{29} only (Ingram et al., 1983) or along with a GA_{29}-catabolite (Ingram et al., 1984). On the basis of the above results, it was concluded that the Le gene in peas controls the 3β-hydroxylation of GA_{20} to GA_1 which is responsible for its stem elongation (Potts et al., 1982; Potts and Reid, 1983; Ingram et al., 1983).

Gaskin et al. (1985) have, however, reported that dark-grown shoots of both the tall and dwarf cultivars of peas had identical amounts of GA_1 and there was little qualitative difference in the different GAs present in these plants. They suggest that the higher levels of GA_{29} present in dwarf plants may prevent the detection of GA_1 in these plants.

McComb and McComb (1970) performed reciprocal grafts between the tall variety Alaska and the dwarf Progress 9 and showed that neither the roots nor the mature leaves determined shoot phenotype in peas. They concluded that growth was not controlled by gibberellins arriving from the roots or from mature leaves and suggested that in plants homozygous for _le_ control of stem elongation did not lie in the pathway of gibberellin biosynthesis but possibly in the subsequent events leading to growth.

On a slightly different note, Chailakhyan (1979) reported the presence of a natural inhibitor, quercetin-glycosyl-coumarte in leaves of peas and found negative correlation between the stem height and inhibitor content. Kefeli _et al_. (1973) have also suggested that inhibitors play a role in inhibition of stem growth in dwarf pea plants.

It thus seems still unclear as to the exact cause of dwarfness in the pea plants.

Materials and Methods

Dwarf pea plants, Pisum sativum L. var. Little Marvel were used as the experimental plant material throughout the present investigation. Seeds were placed in vermiculite and kept in a growth chamber under 14 hours per day of cool white fluorescent and incandescent light (18,000 lux intensity) at a temperature of 23 ± 2°C during the day and 20° at night. One week old plants received a single dose of a commercial fertilizer supplied as an aqueous solution. Ten days after germination, uniformly growing seedlings were treated with 0.8 ug gibberellic acid applied as a 2 ul drop between the terminal leaves which hide the apical bud.

Plant height

The heights of plants were recorded daily for seven days following GA treatment. Heights were measured from the base of the apical bud to the base of the cotyledons.

Extraction of indoles

Plants receiving GA treatment were assayed for extractable indoles at 1, 3, 5 and 7 days following treatment.

Apical portions (20-30 mm of apical tissue of plants showing uniform growth were excised and placed in a beaker over ice. Shoot material (20g) was macerated in prechilled methanol (3 ml/g fr wt) in a Waring blender. The different indoles were separated according to the procedure of Powell (1964) with the exception that extraction at pH 11 was carried out first in order to prevent artifactual formation of a basic indole formed when extraction is made at pH 3 first

(Liu et al., 1978). The extraction procedure is outlined in Appendix A.

Identification of indoles

Indole compounds were identified from plant extracts on the following bases: paper and thin-layer chromatography in several solvent systems, chromatography along with authentic compounds, color reactions with Salkowski and Ehrlich reagents and UV spectra analysis.

a) Paper chromatography. Spots were applied on Whatman No. 1 paper and chromatograms were developed with one of the following systems:

    IAW: Isopropanol: conc. ammonia: water (8:1:1 v/v)
    BuAW: n-Butanol: glacial acetic acid: water (60:15:25 v/v)

b) Thin-layer chromatography. Thin-layer chromatograms were run on plates of silica gel HL(Analtech Uniplates, 0.25 mm thick) or 0.25 mm Polygram Sil G without gypsum (Polygram, Brinkman). For cellulose, Avicel (Analtech) plates were used. The following solvent systems were used:

    i) Silica gel plates.

        CMA: Chloroform: methanol: glacial acetic acid (80:15:5 v/v)
        Protlep: 2-propanol: n-heptane (25:75 v/v)
        BuEA: 1-Butanol: ethanol: 25% ammonia (80:10:10 v/v)
        TEA: Toluene: ethyl acetate: acetic acid (70:25:5 v/v)

ii) Cellulose TLC.

BAW: Benzene: acetic acid: water (8:3:5 v/v)

(Raj and Hutzinger, 1970)

Detection reagents

Indole compounds present on paper chromatograms were detected with the following color reagents.

DMAC: 0.1 g p-dimethylaminocinnaneldehyde was dissolved in 10 ml of concentrated HCl and diluted to 200 ml with acetone immediately before use. The chromatograms were developed by dipping in the reagent, followed by heating in an oven at 65°C for 2.5 min.

DNPH: A saturated solution of 2,4-dinitrophenyl-hydrazine in 2N HCl was mixed 1:1 with ethanol just before use and applied as a spray.

Indole compounds present on thin-layer plates were detected by means of the Ehrlich reagent (Appendix B). Chromatograms were sprayed with 0.25 percent p-dimethylamino-benzaldehyde in a 1:1 mixture of ethanol and conc. HCl and the color was allowed to develop for 16 hr at room temperature.

Chromatography with known indoles:

Plant extracts were first partially purified by chromatography in separate solvent systems: IAW for the acidic and basic fractions, BeAW for the neutral and IBeW for the basic fractions. A spot of the appropriate indole was placed in the plate and chromatographed. Indoles so identified, were then rechromatographed with the TEA solvent system to further purify the acidic and neutral indoles.

Ultra-violet spectroscopy: Suspected indoles were eluted from TLC plates and dissolved in MeOH and the UV spectra of the authentic and suspected indoles were obtained using a Beckman Model 25 recording spectrophotometer.

Quantitative determination of indoles: Quantitative determination of IAA was accomplished by scraping off the Rf zone corresponding to IAA from the thin-layer plate and dissolving in ethanol. The suspension of silica gel (or cellulose) was centrifuged, filtered and subsequently dried over nitrogen. The sample was then subjected to the α-pyrone assay (Stoessel and Venis, 1970). This involved the addition of 0.2 ml of the reagent, consisting of equal parts of acetic anhydride and trifluoroacetic acid, to the sample. The conversion of IAA into indole-α-pyrone was carried out at 0°C for 15 min and the reaction was stopped by adding 3 ml of 90% acetic acid. In the case of blank determinations, 3 ml of acetic acid was first added to the vial containing plant extract, followed by the addition of 0.2 ml of the reagent after 15 min. Fluorimetric determination of indole-α-pyrone was performed after 60 seconds at 449 nm. Blank values were subtracted from the readings and IAA values were calculated from a standard curve prepared using known amounts of authentic IAA and expressed as ng/g fresh weight.

Indole-3-ethanol (IEt) was estimated by applying the concentrated neutral fraction of plant extract on a silica gel HL thin-layer plate and developing in TEA solvent system. The Rf zone for IEt (as compared with co-chromatographed authentic sample and checked under UV) was scraped off into 10 ml of 95% ethanol. The ethanol was evaporated to a minimal volume under vacuum. Two ml of the Salkowski perchloric acid reagent was added to the test solution (1 ml) along with 1 ml of 50 uM IAAld solution and absorbance readings were taken after 5 min at 529 nm against reagent blanks (Vickery and Purves, 1972). Value comparisons were made against a standard curve (Fig. 27).

IET levels were also estimated by spraying thin-layer plates with Ehrlich's reagent and heating for 10 min at 80°C. The plates were then scanned with a Canalco densitometer using a 595 nm filter and peaks recorded on a strip-chart recorder. Estimations were made from a standard curve prepared after plating known amounts of IET.

Experiments with radiolabelled TPP

Apical tissue from plants was ground in a chilled mortar with cold 0.1 M K phosphate buffer, pH 7.0, containing 0.1 M sucrose and centrifuged at 17,300 xg for 30 minutes at 4°. Two ml of the enzyme brei were incubated with an equal volume of the substrate medium containing 0.1 uc of TTP-3-C^{14} at a final concentration of 0.2 mM and specific activity of 0.125 mc/m mole. After 4 hr of incubation in a rotary shaker in the dark at 26°, extractions were made with $MeCl_2$ at pH 7, 3 and 11 to check for the formation of various indole compounds. The $MeCl_2$ extracts were dried down to 5 ml and added to 5 ml of the counting solution. The counting medium consisted of Spectrograde toluene containing the fluors PPO and POPOP mixed with Triton X-100 in a ratio of 7:6. Distilled water was added to the counting vials containing the samples to be assayed. PPO and POPOP were present at final concentrations of 0.4 and 0.005% (w/v), respectively. Radioactivity determinations were made with a Nuclear-Chicago 725 series scintillation system. Quench corrections were made, using external standardization techniques.

Thin layer chromatography of enzyme reaction products were performed with Silica gel HL plates. A 0.5 ml portion of the concentrated extract obtained after 4 hr of enzyme incubation with TTP-3-^{14}C was applied as a 2 cm-wide strip to a silica gel HL thin-layer plate and developed in toluene: ethyl acetate: acetic acid (70:25:5, v/v). After completion of the run, 1 cm-wide bands were scraped from the plate, added separately to 5 ml of methanol, centrifuged (5,000 xg, 5 min) to precipitate the silica gel and then counted as described above.

Enzyme activity:

Tryptophan aminotransferase: The activity of tryptophan aminotransferase (L-tryptophan-α-oxozlutaric acid aminotransferase) was determined according to the procedure of Matheron and Moore (1973). Apical portions of shoot tips were homogenized for 1 min with 2 volumes of 0.5 M borate buffer (pH 8.5) (containing 150 uM chloramphenicol and 1 mM dithiothreiotol) per gram of fresh weight of tissue. The homogenate was passed through eight layers of cheesecloth and centrifuged at 35,000 g for 10 min at 4°C. The resulting supernatant which constituted all of the crude enzyme extract was brought to 25% (v/v) acetone with the addition of ice-cold acetone. After setting in an ice-bath for 10 min, the acetone-crude extract was centrifuged for 10 min. The resultant supernatant was discarded, and the pellet was resuspended in 0.5 M borate buffer (pH 8.5) in a total of one-sixth of the original volume of the crude extract. This suspension was then centrifuged at 40,000 g for 10 min and the resultant light golden colored supernatant was used as the acetone precipitated enzyme fraction.

The enzyme was further purified by loading on a 2.5 x 45 cm Sephadex G-200 column and eluting with 0.1 M phosphate buffer (pH 8.0) at a flow rate of 8 ml per hour. Five ml fractions were collected and their OD's recorded at 280 nm.

The standard reaction mixture for determination of tryptophan transaminase activity contained 0.5 ml of enzyme extract and 2.5 ml of 0.1 M borate buffer (pH 8.5) containing 0.1 u mole of pyridoxal phosphate, 40 u moles of D,L-Tryptophan, and 20 u moles of -ketoglutaric acid. The reaction mixtures were incubated for 60 min at 30°C in rubber-stoppered 10 ml test tubes. Reaction mixtures minus -ketoglutaric acid or with boiled enzyme served as blanks.

The enol-borate buffer assay of Lin et al. (1958) was used to assay the formation of indolepyruvic acid during the reaction. The enol tautomers of the aromatic -keto acids combine instantaneously and reversibly with borate to form complexes of a mixed ester-anhydride structure, which in turn displaces the keto-enol equilibrium in favor of the enol tautomer (Matheron and Moore, 1973). The normally unstable keto acid (Kaper and Veldstra, 1958) is trapped as the more stable enol tautomer and can be read at 328 nm (Knox and Pitt, 1957).

Formation of glutamic acid during the reaction was also followed by performing thin layer chromatography (Matheron and Moore, 1973).

This involved the termination of the reaction by addition of 2 drops of conc. HCl. The reaction mixtures were then placed in a boiling water bath for 5 min to precipitate protein. They were then centrifuged at 5,000 g for 5 min and the resulting supernatant collected. Spots were applied on a silica gel G thin-layer plate and co-chromatographed with authentic D,L-glutamate. Plants were developed in 1-butanol-glacial acetic acid-water (80:20:20 v/v/v) solvent system. The chromatograms were dried and sprayed with ninhydrin reagent (Waldi, 1965) (Appendix E). Plates were then heated to 110°C for 10 min. Spots corresponding to authentic glutamate were scraped and dissolved in 5 ml of 70% (v/v) ethanol. The suspension was then centrifuged at 5,000 g for 5 min and the supernatant collected and absorbance measured at 575 nm.

IPyA decarboxylase; IAAld dehydrogenase; IAAld reductase

Apical portions of shoots were homogenized with 0.05 M Tris-HCl buffer (pH 7.5) in a Waring Blender for 1 min at 4°C. One ml of buffer was used for 1 g of seedling. The homogenate was filtered through eight layers of cheesecloth and the filtrate was centrifuged at 10,000g for 30 min in a Sorvall centrifuge at 4°C. The supernatant was tested immediately for enzyme activities. Assays were made for enzymes in three pH ranges with the following buffers: 0.1 M Na-Phosphate-citric acid (pH 2.5 - 7.5), 0.1 M phosphate (pH 6 - 8) and 0.1 M Tris-HCl (pH 7.2 - 9).

IPyA decarboxylase activity was determined according to the method of Gibson _et al_. (1972). This involved incubating 5 nM indole-pyruvic acid in 12.5 ml of Tris-HCl buffer at pH 8 in the presence of the enzyme (5 ml) to 1 hr at 35°C in the dark with constant shaking. At the end of the reaction period, the indoleacetaldehyde formed was extracted with methylene chloride and dried down in a rotary evaporator. The sample was then applied on a silica gel HL thin-layer plate and developed in TEA solvent system along with known IAAld as marker. The Rf zone corresponding to IAAld in this solvent system (0.75) was scraped off, eluted in methanol and checked with the modified Salkowski reagent (Appendix B) for color reaction. Absorbance readings were made at 529 nm with a Beckman spectrophotometer in the presence of 0.4 mM indoleethanol according to the procedure of Vickery and Purves (1972).

IAAld dehydrogenase: The supernatant from the 10,000 g centrifugation was assayed for the aldehyde dehydrogenase activity (indol-3-yl acetaldehyde-NAD oxidoreductase) according to the procedure of Liu et al. (1978). The substrate used for the assay was the free-form indoleacetaldehyde prepared according to Brown and Purves' method (1976).

Indole-3-acetaldehyde was purchased as the bisulfite adduct from Sigma Chemical Company and stored desiccated at -20°C. The IAAld bisulfite was dissolved in a volume one-fifth that of the volume desired (5X final incubation conc.) and the pH raised to approximately 10 with saturated Na_2CO_3. The solution was allowed to stand for 5 min and the free indoleacetaldehyde was then removed by three extractions with equal volumes of anhydrous diethyl ether. The ether extracts were combined with an appropriate volume of water and the ether was removed under vacuum in a rotary evaporator. The final concentration of free indoleacetaldehyde was determined by measuring the absorbance at 280 nm and applying Beer's law with $E_M^{280} = 5400$ liter mol^{-1} cm^{-1}.

The activity of the enzyme was determined in the crude plant extract by running an assay containing 4 ml of the supernatant and 8 ml of the substrate containing 5 nM indoleacetaldehyde with 0.1 mM NAD. After 1 hr at 25°C the reaction was stopped by bringing the volume up to 100 ml with distilled water. The pH was adjusted to 11 with Na_2CO_3 and was extracted twice with methylene chloride. The aqueous phase was adjusted to pH 3.0 with H_3PO_3 and was extracted twice with methylene chloride. The acid fractions were combined and dried in a rotary evaporator to a minimal volume. The extract was applied onto a Silica gel HL thin-layer plate (Analtech) and developed on TEA or EIA (ethylacetate: isopropanol: ammonia, 45:35:20 v/v) solvent system. The IAA spot was localized by spraying with the Ehrlich reagent or matched with authentic IAA spot under UV.

IAAld reductase: Supernatant from the 10,000 g centrifugation was assayed for IAAld reductase activity according to the procedure by Liu et al. (1978). For the enzyme activity, the reaction mixture contained 4 ml of enzyme and 1 ml of substrate solution at various pH values. The substrate solution contained 0.1 M NADH and 5 mM of IAAld. Reaction was run at 37° for 30 min initially but later modified and run for 5 min with partially purified enzyme according to the procedure of Brown and Purves (1980). At the end of the reaction period, the volume was brought up to 100 ml with distilled water. The pH was adjusted to 11 with Na_2CO_3 and extracted twice with methylene chloride. The aqueous fraction was adjusted to pH 7.0 and extracted twice methylene chloride. The neutral fractions were combined, dried in a rotary evaporator to a minimal volume, applied on a Silica gel HL thin-layer plate and developed in TEA or EIA solvent system. The Rf spot corresponding to IEt (Rf in TEA = 0.28), as determined by UV scan and color reaction with Ehrlich reaction, was scraped off and dissolved in ethanol. Color was developed with Salkowski perchlorate reagent (with 0.4 mM IAAld) and read at 529 nm. Colored spots on the TLC plate were also scanned with a Canalco Model K densitometer and ug/g fresh weight IET formed were calculated from a standard curve prepared by plating known amounts of IEt under similar conditions.

Partial purification of IAAld reductase and IAAld dehydrogenase

Ammonium sulfate precipitation of the crude enzymes showed localization of the proteins at 50% saturation. The precipitate was allowed to settle for 30 min and then centrifuged at 10,000 g for 10 min. The precipitate was dissolved in PO_4 buffer (pH 7.5) and dialysed overnight at 4°C. The dialysed fraction was then applied to a Sephadex G-25 column (25 cm x 2.5 cm) and eluted with elution buffer. All fractions were assayed for the dehydrogenase activity with IAAld as the substrate and NAD as the co-factor for the dehydrogenase and NADH as the co-factor for the reductase. Fractions showing activity were pooled together and concentrated with an Amicon Ultrafiltration cell, Model 202, with an Amicon Diaflo Ultrafiltration membrane, UM2. The media was concentrated down to 5 ml. The extract was then applied to a column (Pharmacia Fine Chemicals K25/45) maintained at 4°C by a water circulator and packed with Sephadex G-100 swelled in phosphate buffer, pH 5.9, along with 0.02% NaN_3 as an antimicrobial agent. The column was fitted with a flow adaptor and a 4-way valve (Pharmacia A-25 and LV-4). Two ml of the extract was injected through the valve followed by 1 ml of 10% sucrose solution. Three ml fractions were collected and assayed for both dehydrogenase and reductase activities. Column dimensions, bed height and volume, flow rate, operating pressure, void volume and column packing procedure are described in Appendix F.

IAA oxidation

In order to check the activity of IAA oxidase/peroxidase system, enzyme brei was prepared according to the method of Seevers et al. (1971). Fifteen gm of apical tissue were harvested. Tissue was homogenized in 0.1 M K-phosphate buffer (pH 8.0) for 1 min in a Waring blender. The resultant brei was strained through 8 layers of cheesecloth and centrifuged at 20,000 xg for 20 min. The supernatant was collected and subjected to 50-90% NH_4SO_4 precipitation. The protein collected was dissolved in K-phosphate buffer, pH 8.0, and applied to a G-25 Sephadex column (23 cm x 2.5 cm).

Column chromatography was carried out at 4°C with a flow rate of 12 ml per hour. The elution profile was monitored by enzyme assay using O-dianisidine as a substrate (Seevers et al., 1971) and by protein estimation with light absorbance at 280 nm using a UV-monitor (Bio-Red Model 1300) attached with a strip chart recorder. Oxidase activity was also monitored directly from the elution volumes with IAA as a substitute (Ueng and Daly, 1985).

The colorimetric assay of peroxidases was carried out at 25°C. The reaction mixture consisted of 1.8 ml of 20 mM sodium acetate buffer (pH 5.0); 0.05 mL of O-dianisidine (1% in absolute methanol; 0.2 ml of 30 mM H_2O_2; and 0.1 ml of enzyme solution. An equivalent volume of buffer was substituted for H_2O in the reference cuvette. Absorbance was recorded with a Beckman spectrophotometer at 470 nm.

Peroxidase-mediated IAA oxidation was measured at 20°C in 2.0 ml of reaction solution containing 0.2 m mol acetate bufrer, pH 4.5, 1.0 u mol IAA, 0.2 u mol 2,4-DCP, 0.2 u mol $MnCl_2$ and enzyme. The IAA remaining in the reaction mixture after the incubation period was measured using Salkowski reagent (Gordon and Weber, 1951) and measured at 525 nm with a Beckman spectrophotometer.

IAA oxidase inhibitors

Apical portions of shoots were homogenized in cold 0.1 M K-phosphate buffer, pH 6.0 (5 ml per g fresh weight of tissue in a Waring blender). The homogenate was filtered through 4 layers of cheesecloth, then centrifuged at 20,000 xg for 20 min. The supernatant was collected and heated for 10 min in boiling water. The extracts were cleared by centrifugation at 16,000 g and the clear, yellow supernatant was used as a source of IAA oxidase inhibitor (Gelinas and Postlethwait, 1969).

Phenol estimation

Total phenol content of the inhibitor preparation was estimated by mixing 0.1 ml of undiluted inhibitor preparation with 5 ml of the Lowry reagent (Lowry *et al.*, 1951). The tubes were allowed to stand for 45 min at room temperature, then 0.5 ml of the Folin phenol reagent was added. The tubes were allowed to stand for another 15 min, then were diluted with deionized water to 10 ml total volume. The percent transmittance at 750 nm was recorded and converted, by means of a standard curve, to ug equivalent of chlorogenic acid (Fig. 23).

Phenol content was also estimated according to the procedure of Amorin *et al.* (1976). Fresh tissue was extracted with absolute ethanol (5 ml per gram of fresh weight). The extract was heated gently for 20 min and then allowed to stand for 24 h at 4°C for protein precipitation. The precipitate was removed by centrifugation at 3000 g for 15 min. Total phenol determinations were made by using 0.5 ml aliquots of the supernatant mixed with 5 ml of deionized water followed by the addition of 1 N Folin-Ciocalteau reagent and immediate mixing. Three minutes later, 0.5 ml of a saturated Na_2CO_3 solution was added and mixed thoroughly. The resulting blue complex was measured 2 hr later at 660 nm.

Bioassays

To confirm the biological activity of IEt, three different bioassays were used: *Avena* straight growth test, cucumber root inhibition and *Coleus* petiole abscission tests (Valdovinos and Perely, 1966).

Avena coleoptile straight growth test

Avena seeds (Avena sativa L. var. Spear) were dehusked and soaked in distilled water for 2 hr. The seeds were then placed on petri plates over filter papers that had been pre-washed with deionized water to remove inhibitors. The petri dishes, containing the seeds, were placed in a growth room in dim red light (25 watts) for 22 hr. The room temperature was maintained at about 22°C. All subsequent manipulations were performed in the dark under a green, safe light. After 2 hrs of darkness, the seeds were planted in wet, sterilized, coarse sand. The seed trays were then placed in a growth chamber maintained at 22°C and R.H. of 80-85% in total darkness for approximately 48 hr. The coleoptiles were then decapitated and after 2 hr, segments were collected and placed into test tubes containing incubation media. After 48 hr, sections were removed and measured under a dissecting microscope.

The assay solution was made from an 0.05 M citrate phosphate, buffer solution (pH 5) containing 2% sucrose, 0.005% Tween 80 and varying concentrations of IAA or IEt.

Cucumber root inhibition test

Cucumber seeds (Cucumis sativa L. var. Straight Eight) were surface-sterilized with quarter strength Chlorox for 5 min and then thoroughly washed with deionized water. Seeds were placed on petri plates over moist filter paper and allowed to germinate in the dark in a growth chamber at 23°C. Seedlings with radicles averaging 5 mm in length were placed in small petri plates on filter paper with 5 ml of the varying concentrations of IEt or IAA. The petri plates were placed in a growth chamber in the dark at 23°C. After 48 hr the seedlings were removed and the lengths of the primary roots were measured.

Coleus petiole abscission test

Coleus *blumei* Benth var. Scarlet Rainbow plants grown under normal greenhouse conditions were used in the abscission test. The procedure described by Valdovinos and Perley (1966) was used during these experiments. This involved removing the lateral buds at the fourth mode of each plant followed by deblading 3 cm distal to the abscission zone. Aqueous solutions of the chemicals (IEt and IAA) were applied in glass capillary tubes inserted into the debladed petioles. Solutions were replaced as needed. The number of days for 50% of the petioles to abscise was recorded for each treatment. Debladed petioles of control plants received deionized water. To prevent possible conversion of IEt to IAA, dimedone (10 uM) was used as an aldehyde trap and was used along with IEt treatments.

Extraction of growth-promoting substance from GA-treated plants

Dwarf pea plants treated with GA_3 were used to investigate the presence of a growth-promotive substance following treatments. Extracts were made from plants as per the schedule outlined in Appendix G. Extractions were made for several days following GA_3 application. The aqueous extracts were applied as a 2 ul drop between the leaves which cover the apical bud of dwarf pea plants. Plant height measurements were made as described before. The internodes were numbered from the base up, with internode number 1 being considered as the first internode above the cotyledonary node. The fraction obtained as the aqueous solution remaining after n-butanol extraction at a neutral pH was called Aqueous Fraction 1. The fraction remaining after ethyl acetate extraction was designated as Aqueous Fraction 2. Extracts (2.5 ml) were applied on petri plates on which cucumber radicles were allowed to grow as per the protocol described for the cucumber radicle bioassay.

Results

Plant height

Heights of dwarf pea plants treated with 0.8 um GA_3 were recorded at daily intervals up to 7 days after treatment. It was observed that gibberellin treatment results in a marked increase in height of plants so that after 7 days, the treated plants were 134% taller than the control plants (Fig. 1). The maximum difference in height was six days after treatment (142% over controls) but results suggest that the initial surge in stem growth started about 3 days after treatment with GA_3. Thus, while treated plants were 34% taller than controls three days after treatment, they were 126% taller after four days.

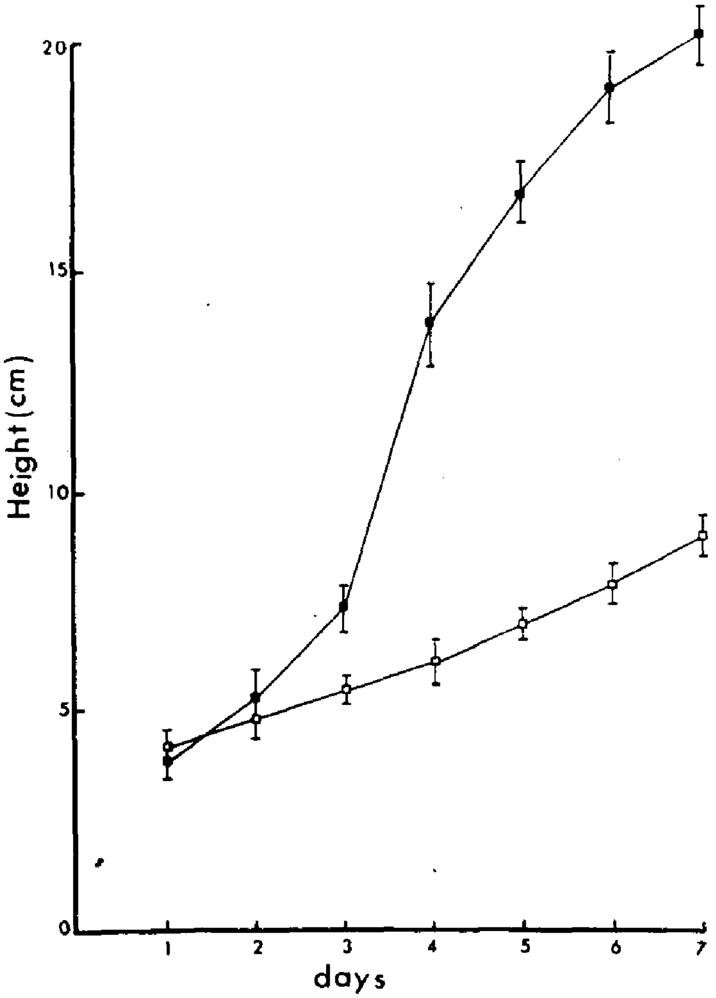
Figure 1. Effect of GA_3 on the height of dwarf pea plants.

abscissa = days after treatment

ordinate = height of plants in cm.

control plants = open circles

GA_3 treated plants = closed circles



Identification of indoles:

Indoles were examined from the dried down methylene chloride extracts obtained after partitioning at three different pHs (11, 7 and 3) according to the procedure of Liu *et al*. (1978) as modified from Powell (1964).

Paper chromatography:

Extracts obtained from plant tissue were subjected to ascending paper chromatography in isoproponol: ammonia: water (8:1:1, v/v) and n-Butanol: acetic acid: water (60:15:25, v/v) solvent systems. The IAW solvent system proved to be better and was able to resolve IAA and ILA in the acidic fraction and IAAld and IEt in the neutral fraction. The BAW solvent system proved to be unsatisfactory for the neutral indoles. In neither of the two systems was tryptamine detected.

Thin-layer chromatography:

Thin-layer chromatography was performed with several solvent systems. Table 1 shows that the different indoles that could be identified were IAA and IPyA in the acidic fraction and IAAld and IEt in the neutral fractions. Traces of ILA were observed in several solvent systems. The detection of IPyA was very difficult and could only be detected clearly in the cellulose TLC plates with BAW or TEA solvent systems. Best results were obtained when preheated silica gel HL plates were used with TEA as the solvent system. In most cases, the TLC plates were dried and sprayed with the Ehrlich reagent to detect the indoles. Co-chromatography with known indoles also helped in identification. Rf zones were also located under UV light, scraped off and dissolved in methanol to test for color reactions with the Salkowski reagent. Results show fairly characteristic color reactions for the indoles obtained from pea plants (Table 1).

Table 1. Rf values of indoles and their color reactions.

Compound	Rf in solvent system					Color reaction	
	1	2	3	4	5	Ehrlich	Salkowski
ILA	.33	.02	.23	.07	- -	Purple	Orange
IAAld	.88	.44	.86	- -	.75	Purple	Pink
IEt	.89	.50	.93	.75	.29	Purple	Yellow
IAA	.96	.38	.22	.75	.38	Purple	Crimson
IPyA	.24*	- -	.19	.20	.20	Purple	Crimson

*Decomposition

1 = chloroform - methanol - glacial acetic acid (80:15:5, v/v)
2 = 2-propanol - n-heptane (25:75, v/v)
3 = 1-butanol - ethanol - 25% ammonia solution (80:10:10, v/v)
4 = Cellulose TLC plates: benzene - acetic acid - water (8:3:5, v/v)
5 = toluene - ethyl acetate - acetic acid (70:25:5, v/v)

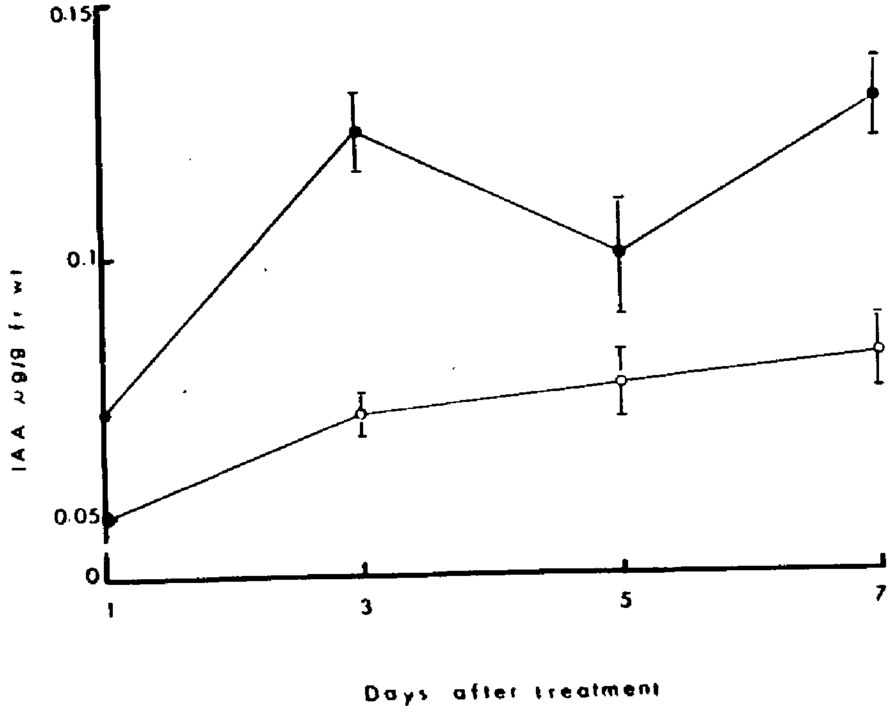
Auxin level:

The extractable auxin level of dwarf pea plants was estimated from the apical tissue of treated and control plants. Results presented in Fig. 2 show that in the dwarf pea plants, the amount of extractable auxin varied from 50 to 81 ng/g fresh weight over the observation period in control plants. However, application of GA_3 resulted in appreciable increases in the auxin level of treated plants. One day after treatment, treated plants had 40% more IAA than controls. Estimations made three days after treatment showed that there was a great surge in IAA production, and treated plants had 125 ng/g fresh weight of IAA which was an increase of 79% over controls. Observations made on the fifth day revealed a slight increase in the auxin level of controls but a large decrease had taken place in GA_3 treated plants. The auxin level at this stage was, however, 35% higher in treated plants. Seven days after treatment with GA_3, pea plants had a much higher content of IAA than control plants (63% more).

Figure 2. Effect of GA_3 on the extractable auxin level of dwarf pea plants.

control plants = open circles

GA_3 treated plants = closed circles



Radiolabelled TpP:

Application of GA to the apical bud of dwarf pea seedlings increased the conversion of D,L-tryptophan-3-^{14}C by enzyme breis from the apical tissue. As seen in Table 2, there was a gradual increase during the 4 hr incubation period in radioactivity of acidic, neutral and basic indole fractions. During a 1 h incubation period, brei from water treated (control) seedlings caused maximum activity in the neutral fraction whereas brei from GA-treated seedlings was associated with maximum activity in the acidic fraction. Activity in the acidic fraction of GA treatments was approximately twice that in controls. Basic fractions of the incubations were much lower in activity than that of neutral and acidic fractions, with little difference observed between controls and GA treatments. Extractions of incubations of controls at 2 hr showed a 25 percent increase in the acidic fraction accompanied by a decrease in the neutral fraction by about 15 percent, when compared to 1 hr incubations. Activity in acidic and neutral fractions of GA treatments increased 20 percent and 15 percent, respectively, during the 1 to 2 hr incubation interval. Activity in basic fractions of both control and GA treatments was increased although it was still lower than that of the acidic and neutral fractions at 2 hr.

Extraction of the incubation mixtures at 4 hr showed approximately twice as much activity in the acidic fraction of GA treatments as in controls. Neutral fraction for GA was 60 percent more active than that of controls whereas little difference in activity was observed in basic fractions of GA and controls.

Table 2. Metabolism of tryptophan-3-^{14}C by pea tissue homogenates following different hours of incubation.

	cpm/g fr wt					
	1 hr		2 hr		4 hr	
Fractions	C	GA	C	GA	C	GA
Acidic	604	1,207	755	1,452	937	1,800
Neutral	1,028	1,100	877	1,268	874	1,401
Basic	387	404	450	550	605	625

Results of thin-layer chromatography of the acidic and neutral fractions are presented in Figs. 3 and 4. A peak of radioactivity was observed to be associated with only one Rf zone in the acidic fraction, corresponding with the Rf of authentic IAA. This zone from incubations of GA treatment had approximately 70 percent more activity associated with it than a similar zone of control incubations. Radiochromatograms of the neutral fraction showed two peaks of activity and these matched the Rf's for authentic IEt and IAAld in the toluene: ethyl acetate: acetic acid solvent system used. The increases in activity for GA treatment over controls were about 40% for IEt and 55% for IAAld.

Characterization of IEt:

The characterization of the suspected IEt obtained after extraction of enzymic reaction products was tested using several procedures. Authentic IEt and the suspected IEt from the incubation medium were compared by TLC using different solvent systems. The compounds were detected on the plates by spraying with Ehrlich reagent. The Rf values were calculated and are presented in Table 3. The Rf zone corresponding to IEt was also identified under UV, scraped off the plate and allowed to react with perchloric Salkowski reagent. This yielded a characteristic pink color that had an absorption maxima at 529 nm (Brown and Purves, 1976). The UV absorption spectrum of IEt was also checked. It was observed that both IEt and the suspected indole had absorption maxima at 275, 282 and 295 nm in methanol.

Table 3. Comparison of Rf value for Indole-3-ethanol and unknown neutral substance extracted from pea seedlings. Determined by silica gel thin layer chromatography.

Solvent	Indole-3-ethanol Rf	Indole from enzyme brei Rf
Chloroform: methanol: glacial acetic acid (80:15:5, v/v)	0.89	0.90
2-Propanol: n-heptane (25:75, v/v)	0.50	0.49
1-Butanol: ethanol: 25% ammonia	0.93	0.92
Toluene: ethyl acetate: acetic acid (70:25:5)	0.28	0.29

Figure 3. Radioactivity scan of TLC plate of acidic fraction obtained after incubating D,L-tryptophan-3-^{14}C in enzyme brei obtained from pea shoot tissue.

abscissa: Rf values

ordinate: counts per minute

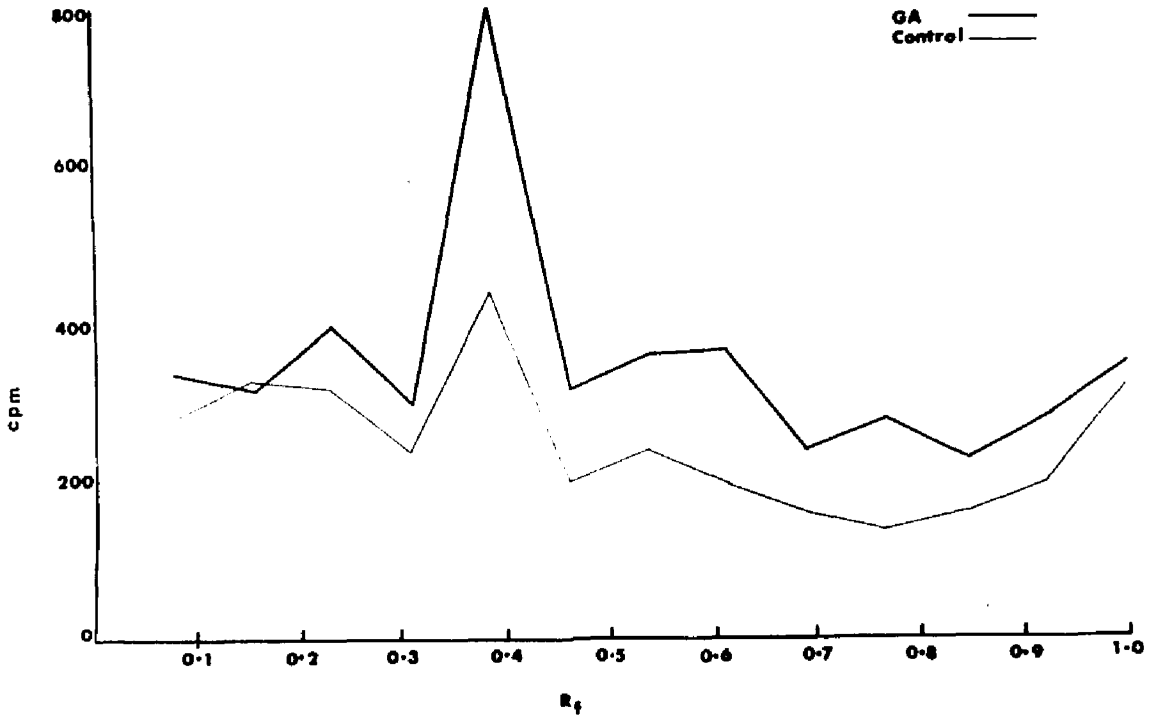
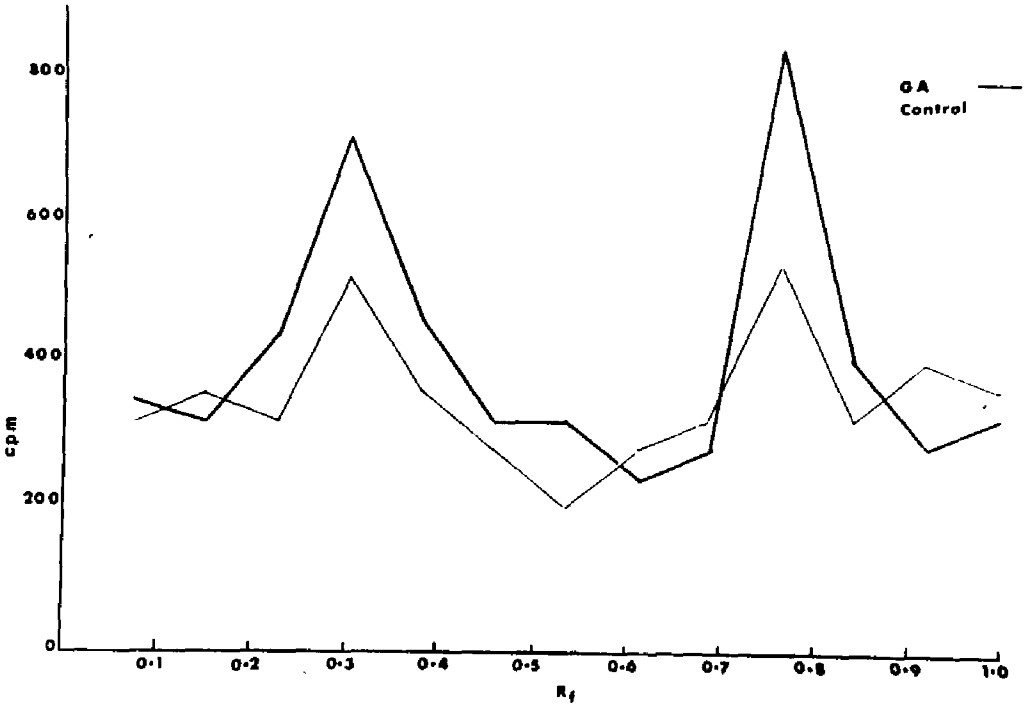


Figure 4. Radioactivity scan of TLC plate of neutral fraction obtained after incubating D,L-tryptophan-3-^{14}C in enzyme brei obtained from pea shoot tissues.

abscissa = Rf values

ordinate = counts per minute



Tryptophan aminotransferase:

Dwarf pea plants treated with GA_3 were harvested 1,3,5 and 7 days after treatment and were assayed for tryptophan transaminase activity.

Purification of enzyme:

The apical portion of the shoots were used as the enzyme source because the apex and primary leaves have been reported to contain the highest enzyme concentration (Truelsen, 1972). The specific activity of the crude enzyme (Table 4) was low and averaged about 0.017 units/mg protein. After purification of the enzyme by acetone precipitation (Matheron and Moore, 1973) the specific activity was increased to an average value of about 0.21 units/mg protein and resulted in a purification factor of 12.3. This preparation was used for pH and temperature optima studies. When the acetone precipitated enzyme was passed through a short G-200 Sephadex column in the presence of 10^{-5} M pyridoxal phosphate (Gibson _et al._, 1972), the specific activity was increased to 1.39 units/mg protein and a resultant purification factor of about 81.7. Any further attempt at purification resulted in loss of activity. The enzyme obtained after gel filtration was used for substrate, kinetics and all other studies.

The influence of pH on the enzyme reaction is depicted in Fig. 5. It shows an optimum at 8.5 with a sharp decline in activity at higher pH values.

To find the temperature optimum for the transamination reaction, the assay was run over a wide range of temperatures. Results presented in Fig. 6 suggests that the optimum temperature for the reaction is 40°C.

Substrate specificity:

Several amino acids and their α-keto analogs were used to test the substrate specificity of the aminotransferase enzyme. In each case, 20 u moles of the keto acid and 40 u moles of the amino acid were used in the reaction mixture with all other conditions being kept constant.

Amino acids:

Results presented in Table 5 show that the enzyme is capable of utilizing all the four amino acids that were tested. The enzyme could utilize both aliphatic and aromatic amino acids. It was also noted that the enzyme was three times as active with D-tryptophan as it was with L-tryptophan. The amounts of IPyA formed from D- and L-tryptophan were 1.06 and 0.35 u moles respectively. A racemic mixture of the D- and L- form yielded 1.18 u moles of IPyA after the incubation period. The above results also show that measurement of glutamic acid also yielded very comparable results and suggests that this could also be used as a test system. The maximum activity of the enzyme was obtained when D,L-Alanine was used as the amino acid substrate.

α-keto acid:

The aminotransferase from dwarf pea plants is capable of utilizing several α-keto acids as substrates (Table 6). Results show that pyruvic acid, α-ketoglutaric acid and oxalacetic acid yielded similar relative amounts of IPyA (1.25, 1.10 and 1.03 u moles respectively) while glyoxylic acid seemed to be a poor substrate and yielded 0.26 u moles (of relative units) of IPyA after the incubation period.

The effects of varying concentrations of tryptophan on the reaction rate is illustrated as a Lineweaver-Burk plot in Fig. 7. The Km for the substrate was calculated to be 4 x 10^{-4} for D,L-tryptophan.

Effect of GA_3 on aminotransferase activity:

The effect of GA_3 application to plants on the tryptophan aminotransferase activity over a one-week period is shown in Fig. 8. It is evident that GA_3 causes an increase in the specific activity of the enzyme after one day following treatment. The maximum increase in activity is observed by the third day (66% over control) after which the specific activity declines sharply. However, the overall activity level of the enzyme was higher (33% over controls) in the treated as compared to control plants at the end of the observation period.

Table 4. Purification of tryptophan aminotransferase

Fraction	Units[1] (umole IPyA)	Protein (mg)	Units/ mg protein	Purification
Extract	5.3	312	.017	1
60% Acetone precipitation	1.47	7	.21	12.3
Sephadex G-200	1.20	0.86	1.39	81.7

[1] Unit = 1 umole IPyA formed/hr

Table 5. Amino acid specificity of tryptophan aminotransferase.

Substrate	Glutamate[1] formed (relative value)	IPyA formed[2] (relative value)
D-Tryptophan	96	90
L-Tryptophan	36	30
D,L-Tryptophan	100	100
D,L-Leucine	83	70
D,L-Alanine	212	182

[1] Reference with D,L-Tryptophan = 1.20 umoles glutamate/hr

[2] Reference with D,L-Tryptophan = 1.18 umoles IPyA/hr

Table 6. Comparative activity with keto acids.

Keto acid	Amino acid product	IPyA formed[1] (relative value)
α-Ketoglutaric	Glutamic	100
Glyoxylic	Glycine	24
Pyruvic	Alanine	114
Oxalacetic	Aspartic	94

[1] Reference with α-ketoglutaric acid = 1.10 umoles IPyA/hr

Figure 5. Optimal assay pH for tryptophan aminotransferase in dwarf pea plants.

abscissa = assay pH

ordinate = A_{328}

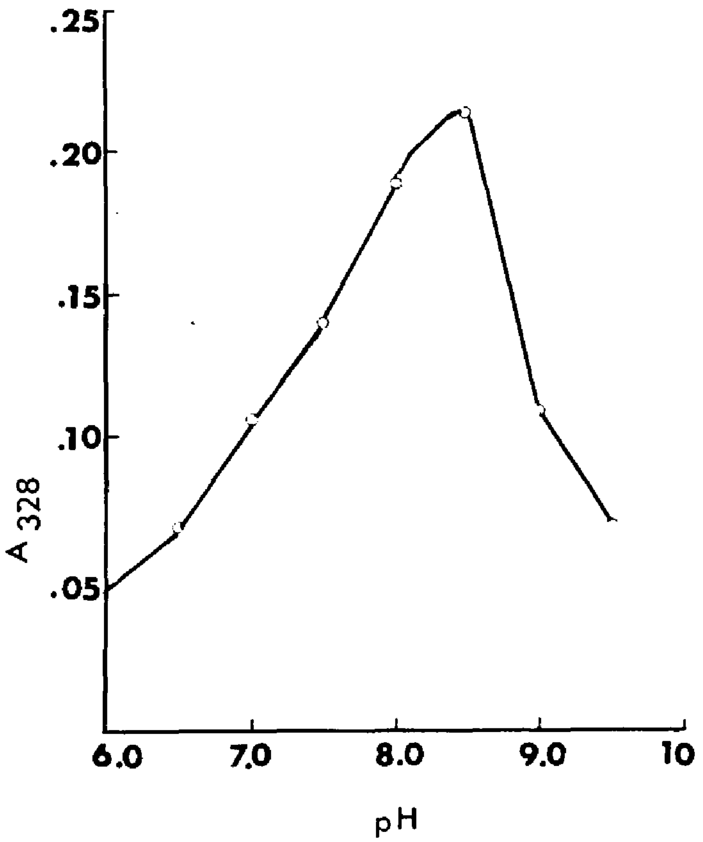


Figure 6. Optimal assay temperature for tryptophan aminotransferase in pea plants.

abscissa = assay temperature (°C)

ordinate = A_{328}

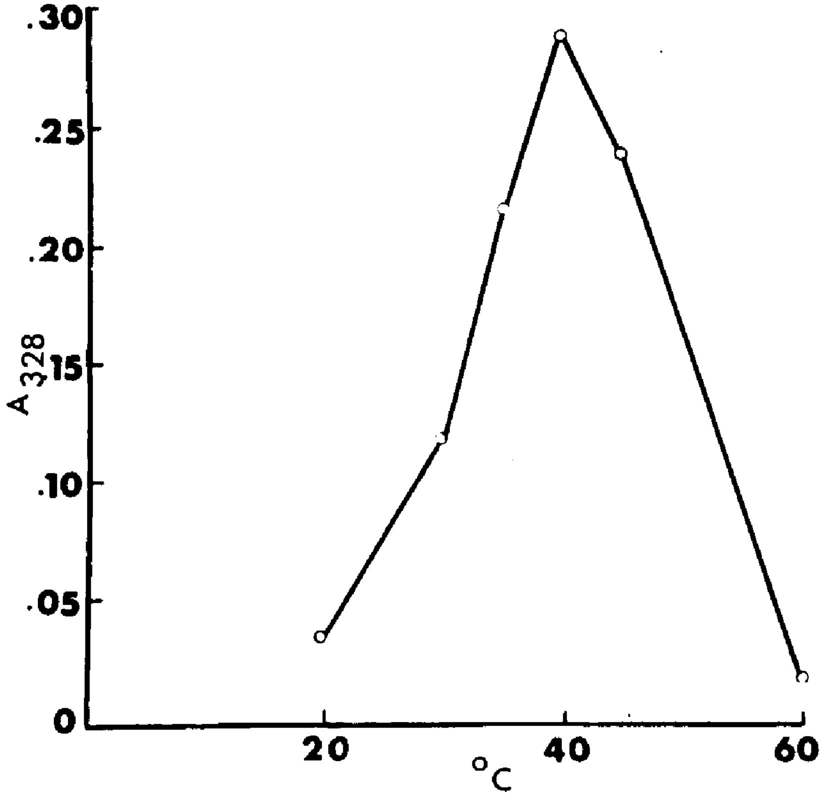


Figure 7. Effect of tryptophan levels on aminotransferase activity. Reciprocal initial velocity versus reciprocal substrate concentrations.

abscissa = reciprocal tryptophan concentration

ordinate = reciprocal initial velocity

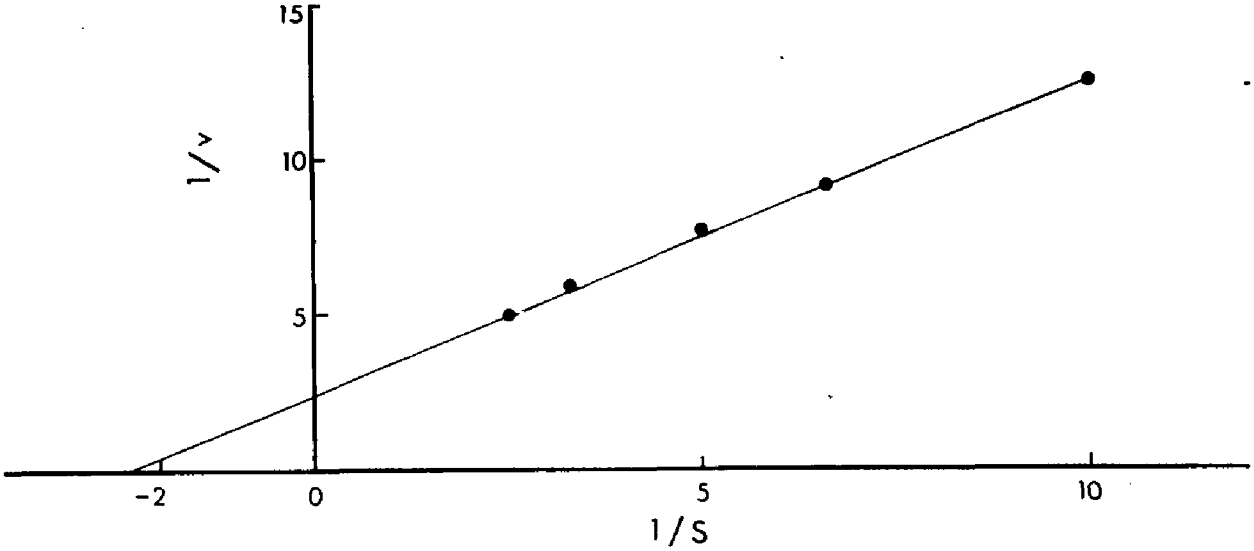


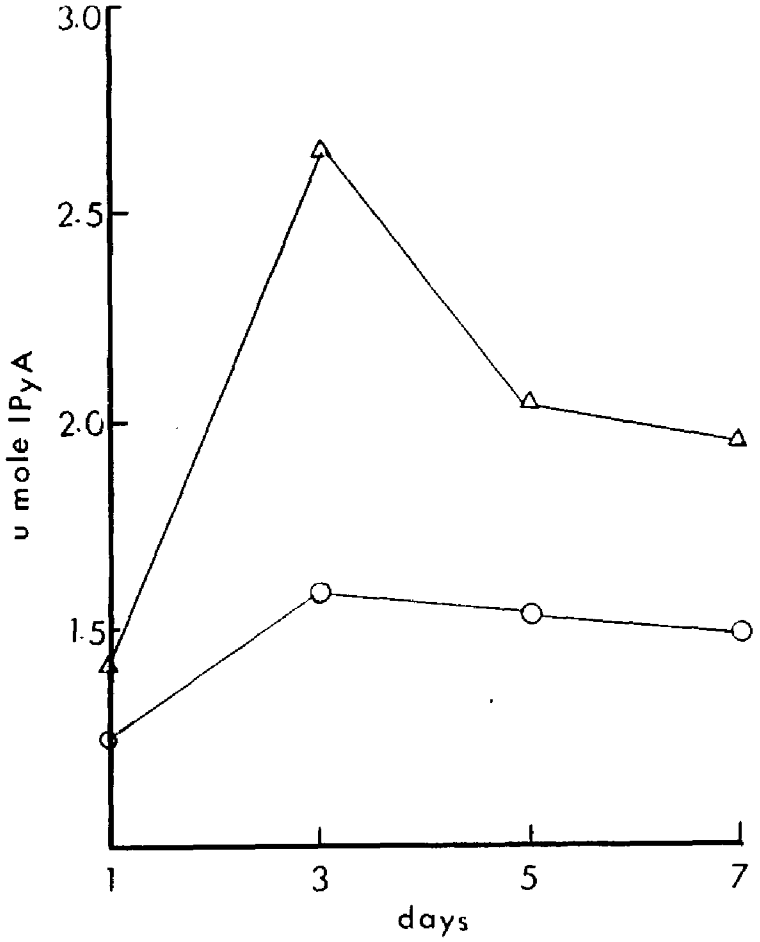
Figure 8. Effect of GA_3 on tryptophan aminotransferase activity in dwarf pea plants.

abscissa = days after treatment

ordinate = specific activity of enzyme (umole IPyA/mg protein)

control plants = open circles

GA_3 treated plants = open triangles



IPyA decarboxylase

The enzyme responsible for converting IPyA to IAAld was found to be present in crude tissue homogenate. The activity of the enzyme was monitored by observing the IAAld formed and reading change in absorbance at 529 nm after color development with the Salkowski reagent. The great instability of IPyA makes this assay extremely difficult to monitor and care had to be taken to subtract readings obtained with boiled enzyme which was considered to be the result obtained due to chemical conversion of IPyA. Also, use of the borate buffer (pH 8.0) helped to stabilize the IPyA and it was observed that use of this buffer could give reproducible results. The enzyme activity was also routinely checked by allowing the reactions to proceed all the way to the formation of IEt (Wightman, 1973).

Results presented in Table 7 show that the enzyme activity showed slight change over the observation period in control plants. In GA_3 treated plants, however, there was a marked increase in the activity of the enzyme three days after treatment. The increase was 33.5% O.D. units in treated plants over controls.

Table 7. Effect of GA_3 on IPyA decarboxylase activity.

Days after treatment	OD_{529} Control	OD_{529} GA_3
1	0.116	0.121
3	0.134	0.179*
5	0.145	0.155
7	0.126	0.139

*Significantly different from control at the 98% level.

Indoleacetaldehyde dehydrogenase

The enzyme responsible for converting IAAld to IAA has been considered to be an oxidase (Rajagopal, 1971) or an NAD-dependent dehydrogenase (Wightman and Cohen, 1966). During the present investigation, it was observed that the NAD-dependent dehydrogenase was present in the precipitate obtained between 40-60 percent ammonium sulfate saturation. The activity of the enzyme was recorded by measuring the IAA formed by measuring the IAA formed by the α-pyrone method at the end of the reaction. Routine observations were also made of the IAA formed by extracting the indolic reaction products with methylene chloride and subjecting to TLC on the TEA solvent system. TLC plates were dried and sprayed with Ehrlich reagent and the IAA spot was observed.

For partial purification of the enzyme, the 50 percent saturated ammonium sulfate precipitate was loaded on to a Sephadex G-100 column and the eluate monitored for dehydrogenase activity with IAAld as the substrate in the presence of NAD. It was observed that the crude enzyme yielded a specific activity of 0.067 units which was increased by a factor of 3.7 when a 50 percent saturated $(NH_4)_2 \cdot SO_4$ precipitate was taken. Passing through a Sephadex G-100 column resulted in the increase of specific activity to 1.66 units which meant about a 25-fold increase in the over all purification of the enzyme (Table 8).

Fractionation of the extract was performed with a chromatography column packed with G-100 Sephadex attached to a UV-monitor to detect protein peaks at 280 nm and a strip-chart recorder. Three ml fractions were collected and tested for the enzyme activity with 5mM IAAld as substrate and 0.1 mM NAD as co-factor and incubated at 37°C for 1 hr. Enzyme activity was measured by reading absorbance change at 525 nm with the modified Salkowski reagent and IAA equivalents were calculated from a standard curve. All the tubes which yielded the characteristic pink color were also tested for enzyme reaction products by performing TLC with the TEA solvent system. They were observed to give positive tests for IAA based on color reaction with Ehrlich reagent and matched Rf value. Results presented in Fig. 9 show that gel filtration yielded three peaks of IAAld dehydrogenase activity and these are arbitrarily denoted as P1, P2 and P3. It was observed that P2 was the major peak of activity. Protein profiles of the above extracts showed three major peaks with a bulk of high molecular weight peptides not associated with the dehydrogenase enzyme. The partition co-efficients of the IAAld dehydrogenase peaks were determined from the formula $K_{av} = (V_e-V_o)/(V_a-V_o)$ as shown in Appendix F. The partition co-efficients of protein standards, which were run at the same time, were used to plot against log of molecular weights (Fig. 10). The molecular weights of the dehydrogenase peaks were read from this graph using the K_{av}'s of the peaks. The molecular weights were found to be 9,200, 22,500 and 40,000 for P1, P2 and P3 respectively.

Effect of pH

The variation in the activity of the enzyme was determined at a pH range of 4.0 to 7.0 (Fig. 11). It was observed that the enzyme had a pH optima of about 5.5.

Effect of GA_3

IAAld dehydrogenase was monitored in plants treated with GA_3 every day for one week after treatment (Fig. 12). It was observed that the enzyme activity in treated plants was very high one day after GA_3 treatment but declined sharply till the third day after which it started to rise again. Thus, one day after treatment, treated plants had 90.6% more specific activity associated with the IAAld dehydrogenase. Two days after GA_3 treatment, the increase in activity was only 33.6% over controls. The enzyme activity tended to be quite similar for treated and controls for the two subsequent days of estimation. Five days after treatment, the enzyme level increased by 34 percent over controls and was 82 percent higher by the sixth day. The activity of the enzyme was quite similar at the last day of observation in control and treated plants.

Effect of dimedone

The activity of the enzyme was also checked by using dimedone as an aldehyde trap. Results presented in Table 9 show that dimedone effectively reduced the conversion of IAAld to IAA at the two concentrations of the chemical used.

Table 8. Purification of IAAld dehydrogenase from dwarf pea shoots.

Fraction	Specific activity (ug IAA/g fr wt/hr)	Purification
Crude extract	0.067	1
50% $(NH_4)_2 \cdot SO_4$ precipitation	0.253	3.7
Sephadex G-100	1.66	24.8

Table 9. Effect of dimedone on IAAld dehydrogenase.

Treatment	ug IAA/g fr wt	TLC plates*
Control	1.75	IAA(+++) IAAld(+)
Dimedone (10 uM)	0.055	IAA(+) IAAld (++)
Dimedone (100uM)	0.015	IAAld(+++) IAA(-)

*Presence (+) or absence (-) of indoles on TLC plates.

Figure 9. Indoleacetaldehyde dehydrogenase activity and protein content of Sephadex G-100 fractionated extracts from dwarf pea shoot tissue.

abscissa = fractions

primary ordinate = IAAld dehydrogenase activity

secondary ordinate = A_{280nm}

IAAld dehydrogenase activity = solid line

protein content = dashed line

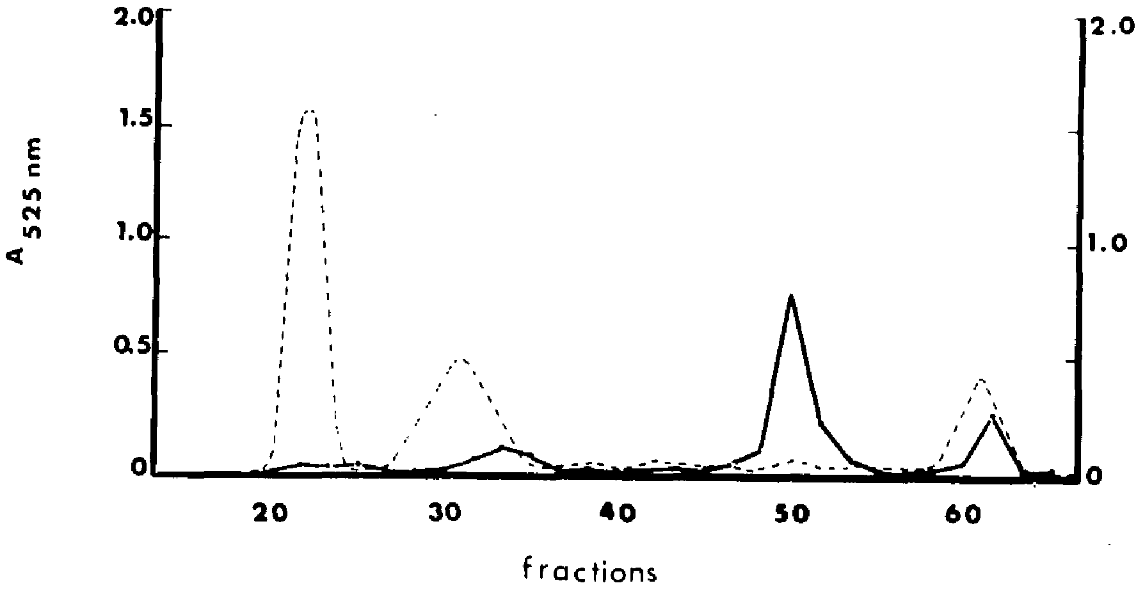


Figure 10. Molecular weight determinations of Indoleacetaldehyde dehydrogenase peaks obtained through Sephadex G-100 fractionations.

abscissa = molecular weights (daltons)

ordinate = K_{av} (partition co-efficient)

Standards:

ribonuclease (open circle)

chymotrypsinogen (open square)

ovalbumin (open triangle)

bovine serum albumin (closed inverted triangle)

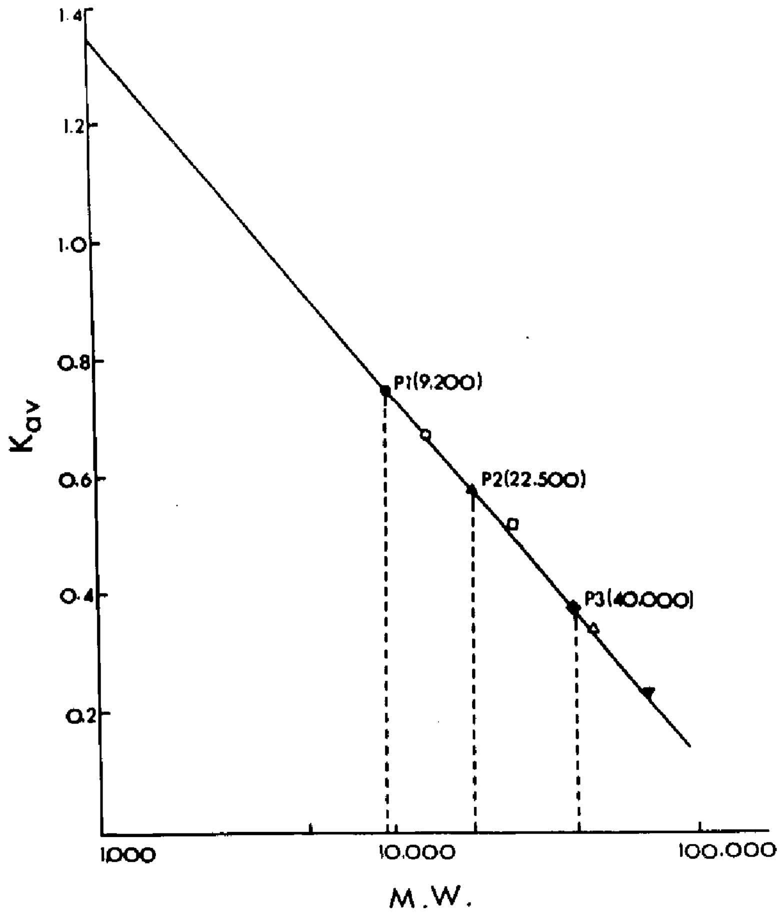


Figure 11. Optimal assay pH for Indoleacetaldehyde dehydrogenase in dwarf pea plants.

abscissa = assay pH

ordinate = IAAld dehydrogenase activity (ug IAA/g fr wt)

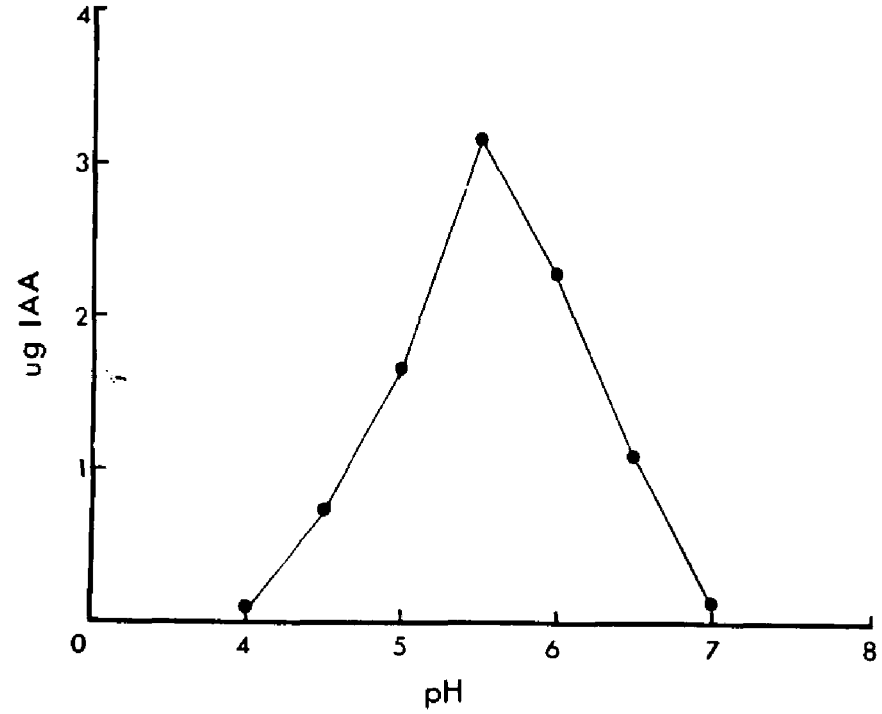


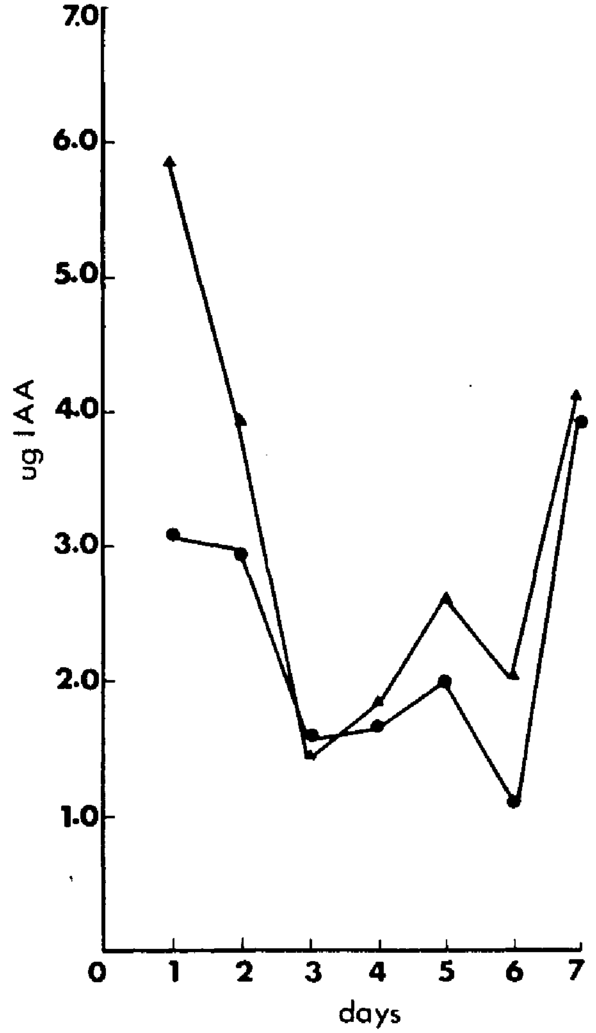
Figure 12. Effect of GA_3 on the Indoleacetaldehyde dehydrogenase levels in dwarf pea plants.

abscissa = days after treatment

ordinate = specific activity of enzyme (ug IAA/g fr wt)

control plants = closed circles

GA_3 treated plants = closed triangles



Indoleacetaldehyde reductase

The supernatant obtained from apical tissue of pea shoots homogenized in 0.05M Tris-HCl buffer (pH 7.5) and centrifuged at 12,000 g for 20 min, was observed to convert IAAld to IEt under the assay conditions described above. The activity of the enzyme was monitored by extracting the neutral indole fraction after completion of assay, and subjecting to TLC in the TEA solvent system. The Rf zone corresponding to IEt was either scraped off, dissolved in methanol, filtered and reacted with the perchlorate Salkowski reagent, or sprayed with the Ehrlich reagent and the colored spot scanned with a densitometer.

Vickery and Purves (1972) have shown earlier that a mixture of IAAld and IEt yields a strong pink color when mixed with the perchlorate Salkowski reagent (Gordon and Weber, 1951). It was shown that the change in absorbance at 529 nm is a function of the ratios of IEt to IAAld (Vickery and Purves, 1972; Percival _et al_., 1973). Fig. 13 shows the absorption spectra of three different ratios of IAAld to IEt developed with the perchlorate Salkowski reagent. The major absorption peak is seen at 529 nm with a small shoulder at shorter wavelengths. The ratio of the shoulder to the peak height was seen to decrease with increases in IAAld:IEt ratios.

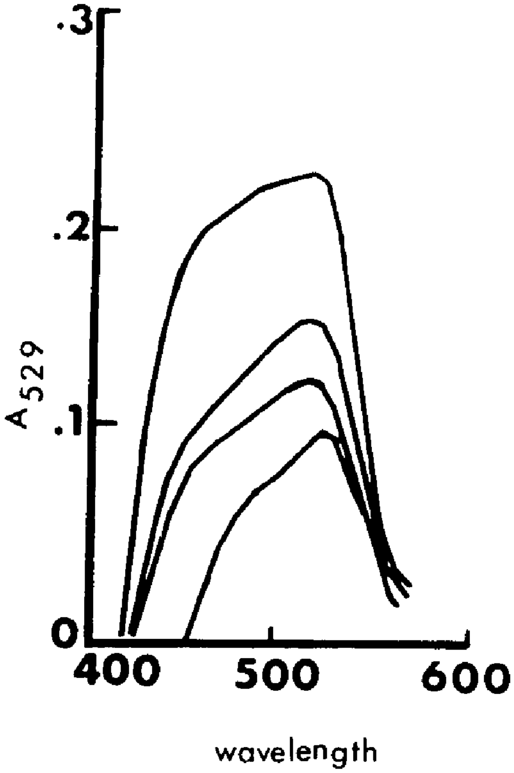
Figure 13. Visible spectra of Salkowski reaction mixtures. Spectra of several mixtures composed of known ratios of indoleethanol to indoleacetaldehyde reacted with Salkowski reagent.

abscissa = wavelength (nm)

ordinate = absorbance

indoleethanol to indoleacetaldehyde ratios:

a = 1.45
b = 0.75
c = 0.30
d = 0.15



The enzyme was purified by loading the 50%$(NH_4)_2 \cdot SO_4$ precipitate on to a chromatography column packed with Sephadex G-100. The proteins were eluted with 0.05M phosphate buffer (pH 5.9) and 3 ml fractions were collected. Protein peaks were monitored by a UV-monitor attachment and recorded automatically on a strip chart recorder. Results presented in Table 10 show that while the crude enzyme yielded a specific activity of 0.035 units, ammonium sulfate saturation increased it to 0.103 - a purification of 3.5 fold. The G-100 Sephadex chromatography was able to increase the activity to 2.4 units which meant an overall purification by 74 times.

Gel filtration results (Fig. 14) show that the IAAld reductase eluted as a single major peak associated with the 105 ml fraction. There were two minor peaks eluting out later so that the peaks were called P1, P2 and P3 with P3 being the major peak of activity. Protein profiles of the above extracts showed three major peaks with a bulk of high-molecular weight peptides coming out close to the void volume. The partition coefficients of the enzyme peaks were determined from the formula $K_{av} = (V_e - V_o)/(V_a - V_o)$ as shown in Appendix F. Known molecular weight proteins (aldolase, bovine serum albumin, ovalbumin, chymotrypsinogen A and ribonuclease A) were chromatographed on the column. The partition coefficients of these standards were determined and plotted (Fig. 15). The molecular weights of the IAAld reductase peaks were read off the graph and were found to be 21,000, 24,000 and 40,000 daltons for P1, P2 and P3 respectively.

Effect of pH

The effect of pH of the assay on the activity of the IAAld reductase was checked at various pH values (Fig. 16). Results show that the enzyme had a pH optima at 7 and a secondary optima at 5.5.

Effect of dimedone and bisulfate

Application of dimedone (100 uM) and bisulfite (50 uM) was found to decrease drastically the enzyme reaction as observed by the absorbance readings at 529 nm (Table 11). Thin-layer plates developed in the TEA solvent system and sprayed with the Ehrlich reagent also did not show formation of IEt by the enzyme.

Effect of substrate concentration

The effect of varying concentrations of IAAld on the reaction rate is illustrated as a Lineweaver-Burk plot in Fig. 17. The Km for the substrate was calculated to be 208 uM of IAAld.

Effect of GA_3

The IAAld reductase level was estimated for 6 days following GA treatment. It was observed that the reductase activity had a general trend of increase over the observation period (Fig. 18). The specific activity of the enzyme in treated plants, however, increased up to the third day after treatment but then declined around the fourth and fifth day after treatment. The activity of the enzyme was again high in the sixth day. As compared to control plants, the specific activity of the enzyme was greater until the third day of observation and the increases were 48, 25 and 47% over controls for 1, 2 and 3 days after treatment. The activity of the enzyme decreased by 14 and 26% respectively on day 4 and 5 and then increased by 34% in day 6 as compared to controls.

Effect of IAA

The effect of different concentrations of IAA on the IAAld reductase level is presented in Table 12. Results suggest that the presence of IAA in the incubation medium negatively affected the conversion of IAAld to IEt by the enzyme. It was observed that increasing the concentration of IAA severely limited the enzyme activity so that while 0.01 mM IAA produced about 27% inhibition, 0.05 mM IAA brought about 50% inhibition of the enzyme activity. Addition of 0.2 mM IAA caused about 77% inhibition of the IAAld reductase activity.

Figure 14. Indoleacetaldehyde reductase activity and protein content of Sephadex G-100 fractionated extracts from dwarf pea shoot tissue.

abscissa = fractions

primary ordinate = IAAld reductase activity

secondary ordinate = $A_{280\ nm}$

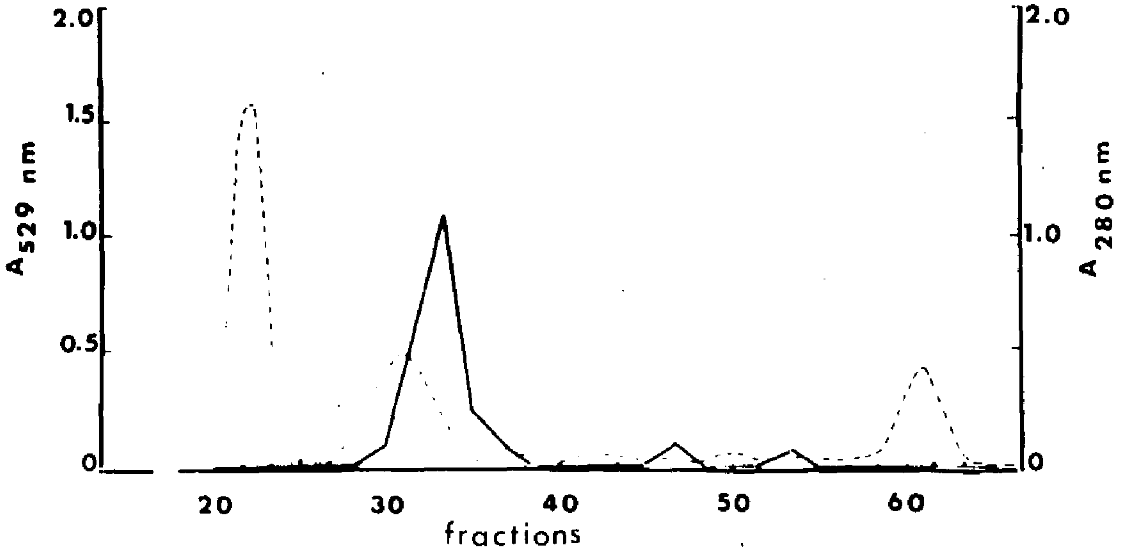


Figure 15. Molecular weight determinations of Indoleacetaldehyde reductase peaks obtained through Sephadex G-100 fractionations.

abscissa = molecular weights (daltons)

ordinate = K_{av} (partition co-efficients)

Standards:

ribonuclease (open circle)
chymotrypsinogen (open square)
ovalbumin (closed triangle)
bovine serum albumin (closed inverted triangle)

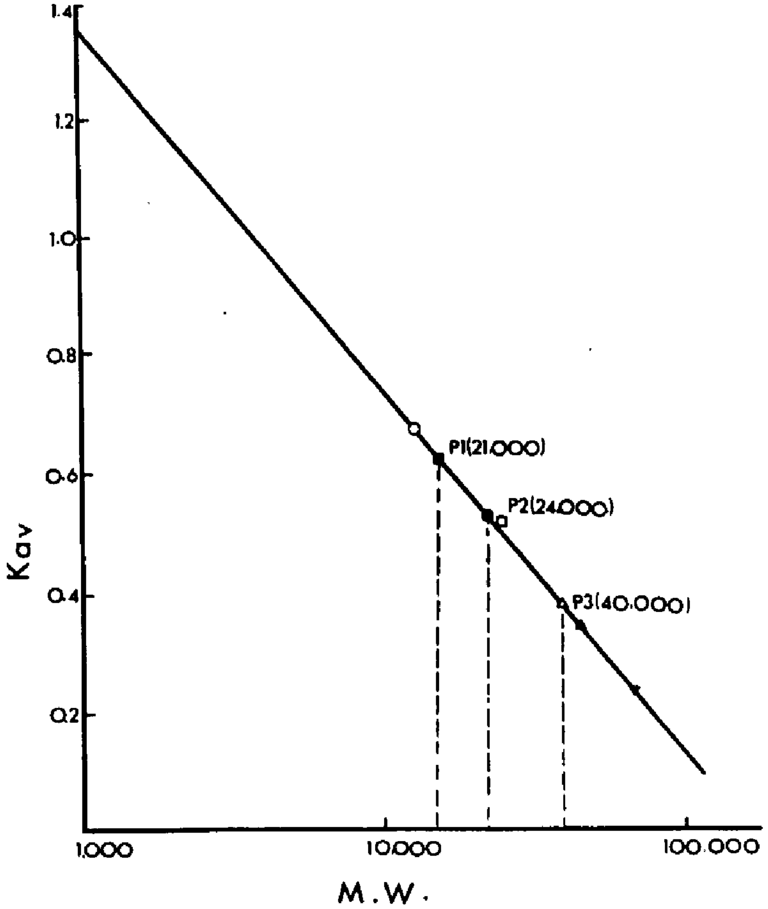


Figure 16. Optimal assay pH for Indoleacetaldehyde reductase in dwarf pea plants.

abscissa = assay pH

ordinate = IAAld reductase activity ($A_{529\ nm}$)

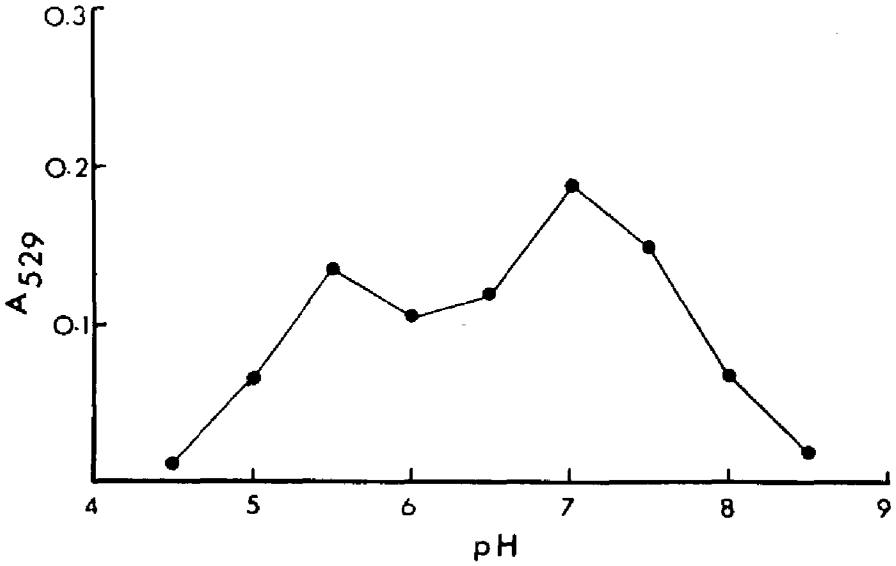


Figure 17. Effect of Indoleacetaldehyde levels on IAAld reductase activity. Reciprocal initial velocity versus reciprocal substrate concentrations.

abscissa = reciprocal IAAld concentrations

ordinate = reciprocal initial velocity

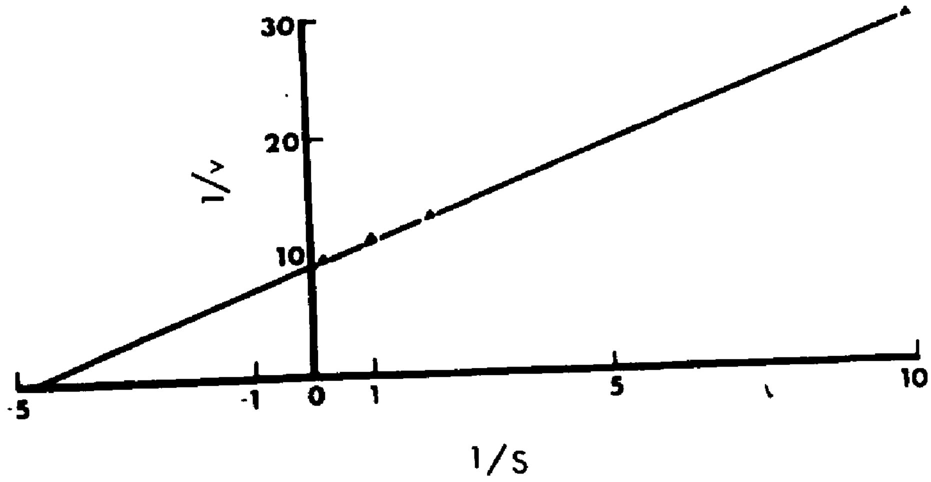


Figure 18. Effect of GA_3 on Indoleacetaldehyde reductase levels in dwarf pea plants.

abscissa = days after treatment

ordinate = IAAld reductase activity (unit activity/mg protein where 1 unit = 0.1/min ΔA_{529})

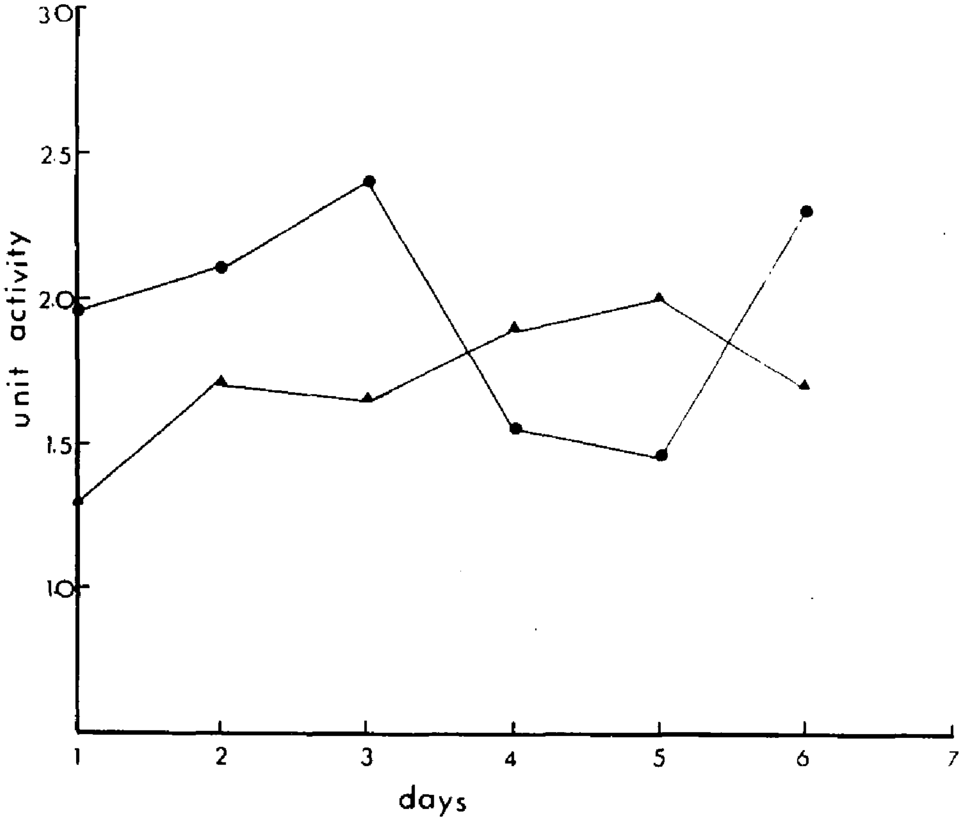


Table 10. Purification of IAAld reductase.

Fraction	Total protein (mg.)	Total enzyme units[1]	Specific Activity	Purification
Crude extract	285	10.16	0.035	1
50% $(NH_4)_2\ SO_4$ Precipitation	81	8.4	0.103	3.45
G-100	2.4	5.8	2.4	74.25

---

[1] = ΔA_{529}/min/ml

Table 11. Effect of Dimedone and bisulfite* on IAAld reductase.

Treatment	A_{529}	TLC plate**
Control	0.278	IET (+++), IAAld (+)
Dimedone	0.156	IAAld (+++)
Bisulfite	0.028	IAAld (+++)

* 50um added to reaction mixture at the start of the reaction.
** Presence or absence of indoles on TLC plates.

Table 12. Effect of IAA levels[1] on IAAld reductase activity.

IAA concs.	A_{529}	% inhibition*
0.01mM	0.216	27.14
0.05mM	0.135	48.21
0.20mM	0.076	77.14

*Determined by calculating against standard reaction mixture which did not get IAA (OD = 0.280).

[1] IAA was added to the reaction mixture at the start of the reaction.

IAA oxidase/peroxidase activity

The fractions obtained between 50 to 90% $(NH_4)_2 \cdot SO_4$ saturation was applied to a Sephadex G-25 column and the elution profile of both control and treated plants was monitored using o-dianisidine or IAA as substrates.

With o-dianisidine; it was observed that the peroxidase activity was localized in a broad peak of the elutant collected between 30 to 80 ml (Fig. 19). This generally coincided with the major protein peak coming out close to the void volume. However, there was a shift in oxidation ability of the different fractions when they were incubated with IAA as a substrate.

Results presented in Figs. 20 and 21 show the oxidation profile as measured as a percentage of IAA remaining in the incubation medium after the reaction period. As compared with o-dianisidine oxidation which is associated with the high molecular weight proteins, the IAA oxidative enzymes are more varied in terms of their molecular weights. Comparison of activity of GA-treated and control plants shows that there is a common peak of activity localized between 35 and 65 ml fractions for both. However, in GA treated plants the oxidase activity is spread over the subsequent volumes collected up to 150 ml of the elutant, whereas control plants had a more well defined peak localized between 70 to 90 ml of the elutant. Both control and treated plants showed a smaller peak around the 150 ml volume.

Results also show very little difference in the IAA-oxidizing activity of the extracts from control and GA-treated plants. While there was a difference in the activity associated with individual fractions, the maximum amount of IAA oxidized was 42% in controls and 44% in GA treated plants.

The 40 to 50 ml fractions were combined, concentrated and passed through a short (25 x 2 cm) tube packed with Sephadex G-200 having a flow rate of 8 drops/min. Results presented in Table 13 show that, with o-dianisidine as a substrate, the protein fraction designated as fraction 6 was associated with maximum oxidizing activity. When IAA was used as a substrate, two peaks of activity were associated with fractions 7 and 9.

The oxidation activity was found to be influenced by the addition of a dihydroxycinuamic acid (caffeic acid) in the reaction mixture (Fig. 22). It was observed that increasing concentrations of caffeic acid (from 1 to 5 uM) increased the time to 50% oxidation (considered as absorbance reading of 0.190 at 529 nm). Results indicate that at the highest concentration of caffeic acid (5 uM), 50% oxidation of IAA occurred at 5 minutes instead of at 2.5 minutes as in controls. After incubation for 10 min, the amount of IAA left still remaining was also slightly higher at the higher concentrations of caffeic acid than in controls.

The oxidation rates of combined 40 - 50 ml and 70 - 90 ml fractions were measured in the presence of caffeic acid (5 uM) and is presented in Table 14. Results show that the presence of caffeic acid in the reaction mixture did not affect the oxidation rate in the 40 - 50 ml fraction. However, the presence of caffeic acid reduced the rate of IAA oxidation in the 70 - 90 ml fraction. Thus, readings taken after 5 min show that while about 77% of IAA had been lost in the absence of C.A., in the presence of C.A. only 57% had been lost.

Figure 19. Sephadex G-25 chromatography of peroxidase activities in dwarf pea shoot tissue with o-dianisidine as substrate.

abscissa = fractions

primary ordinate = A_{470}/min/0.1 ml

secondary ordinate = $A_{280\ nm}$

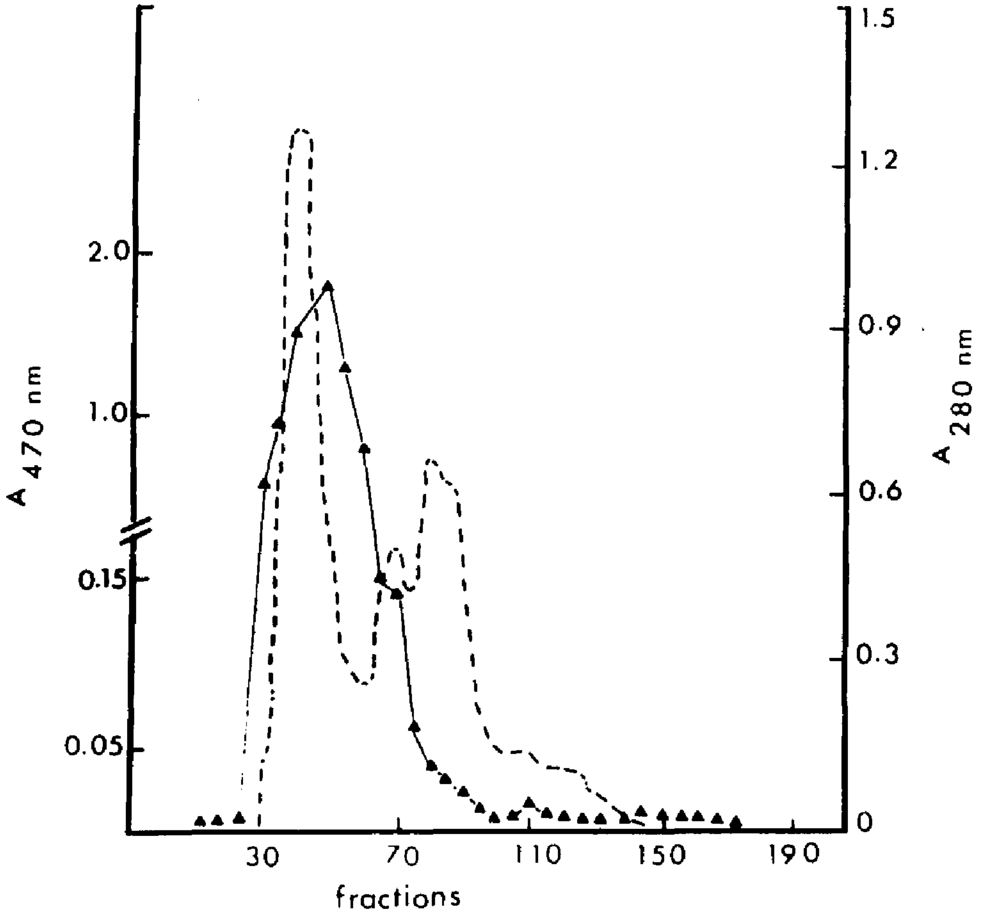


Figure 20. Sephadex G-25 chromatography of peroxidase activity of pea shoots with IAA as substrate.

abscissa = fractions

primary ordinate = % IAA lost (calculated from blanks which were run without enzymes)

secondary ordinate = $A_{280\ nm}$

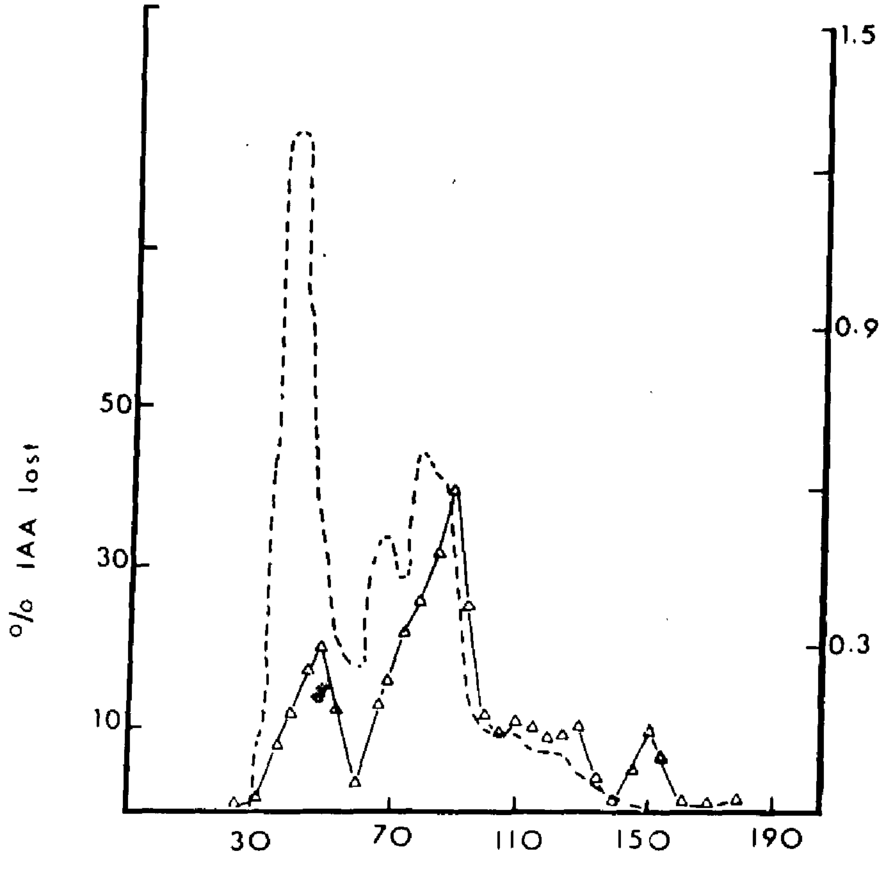


Figure 21. Sephadex G-25 chromatography of peroxidase activity of pea shoots with IAA as substrate following GA_3 treatment.

abscissa = fractions

primary ordinate = % IAA lost (calculated from blanks which were run without enzymes)

secondary ordinate = $A_{280\ nm}$

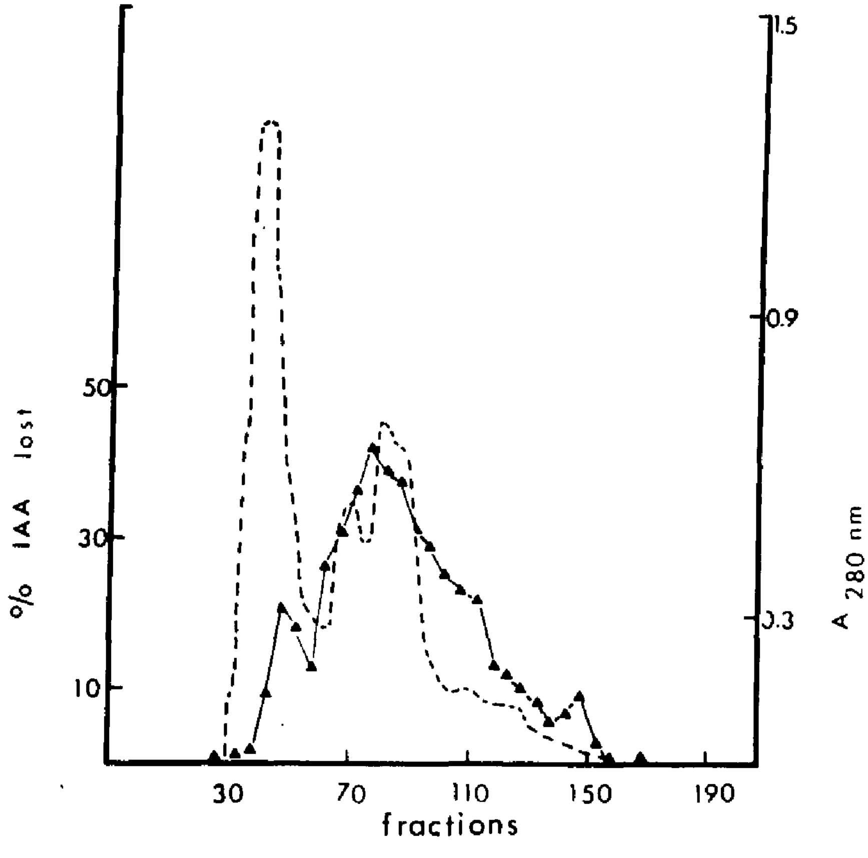


Figure 22. **Effect of caffeic acid levels in the incubation medium on peroxidase-mediated IAA oxidation.**

abscissa = time (minutes)

ordinate = IAA (A_{529})

controls with no caffeic acid (solid squares)

1 mM caffeic acid (open squares)

3 mM caffeic acid (open inverted triangles)

5 mM caffeic acid (closed inverted triangles)

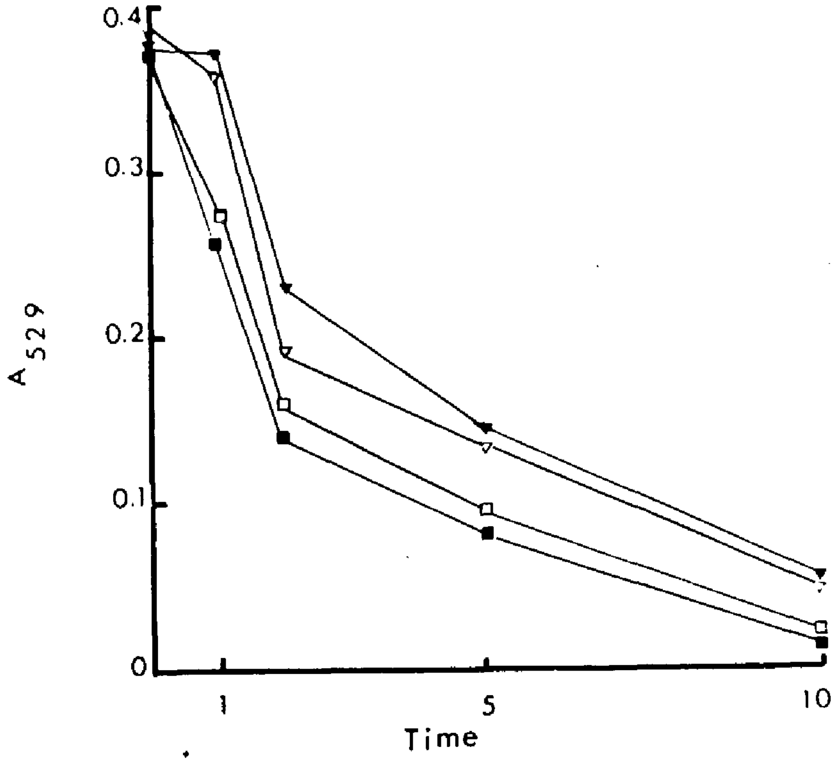


Table 13. Oxidase activity of Sephadex G-25 fractions (40-50 ml) after chromatographing with Sephadex G-200.

Fr. No.	A_{529}[1]	A_{470}[2]
Blank	0.332	- -
4	0.309	0.130
5	0.302	0.696
6	0.185	1.322
7	0.152	0.619
8	0.213	0.771
9	0.162	0.302
10	0.276	0.147

[1] = IAA as substrate (with $MnCl_2$ + DCP)

[2] = o-dianisidine as substrate (with H_2O_2 + acetate buffer pH 4.5)

Table 14. Oxidation of IAA by different fractions in presence of caffeic acid (C.A.).

	OD_{529}			
Minutes	Fraction No.[1] 70-90		Fraction No.[1] 40-50	
	-C.A.	+C.A.	-C.A.	+C.A.
0	.396	.370	.360	.378
1	.315	.342	.310	.306
3	.175	.208	.155	.162
5	.092	.158	.060	.064

[1] 70-90 and 40-50 ml fractions of G-25 column

Phenols

The amount of total ethanol-extractable phenols from shoot tissue was estimated 1, 3, 5 and 7 days after GA treatment. Results presented in Fig. 23 show that there was no significant difference in the total phenol content of tissue between control and GA_3 treated plants. The phenol content of tissue increased about 3-fold by the third day, declined by the fifth and started to rise again when observations were made in both control and treated plants. One day after treatment, based on a standard curve (Fig. 24), the ethanol-extractable phenol was 0.5 ug in controls and 0.8 ug in GA_3 treated plants. Three days after treatment, the phenol level in control plants had gone up to 1.83 ug and in GA_3 treated plants to 2.50 ug - an increase of 54% over the controls. Five days after GA_3 application, the phenols showed a slight decrease in controls (9%) but decreased significantly in treated plants (by 49%). The phenol content of tissue registered a sharp increase in controls and only a moderate rise in treated plants 7 days after GA_3 treatment.

Figure 23. Effect of GA_3 on total ethanol-extractable phenols in the dwarf pea plant.

abscissa = days after treatment

ordinate = ug/g fresh wt (chlorogenic acid equivalents)

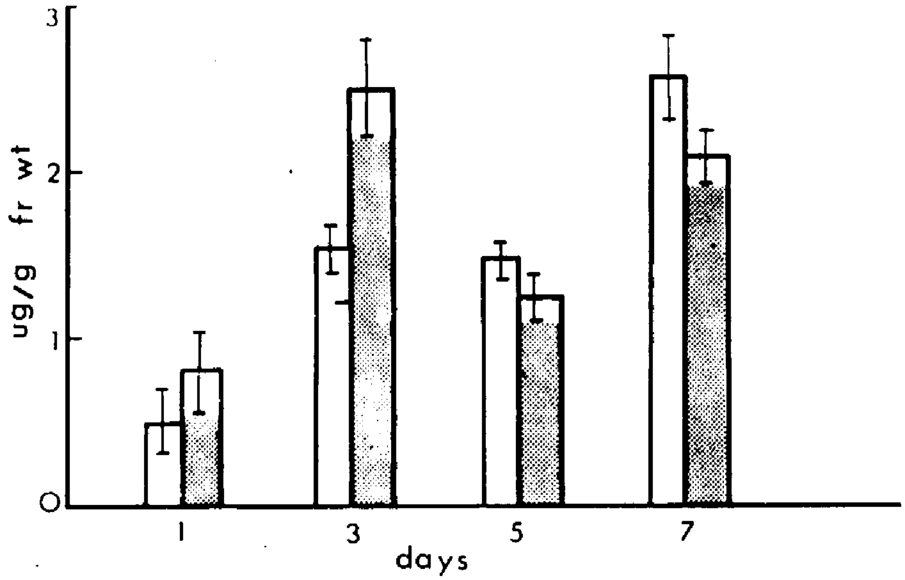
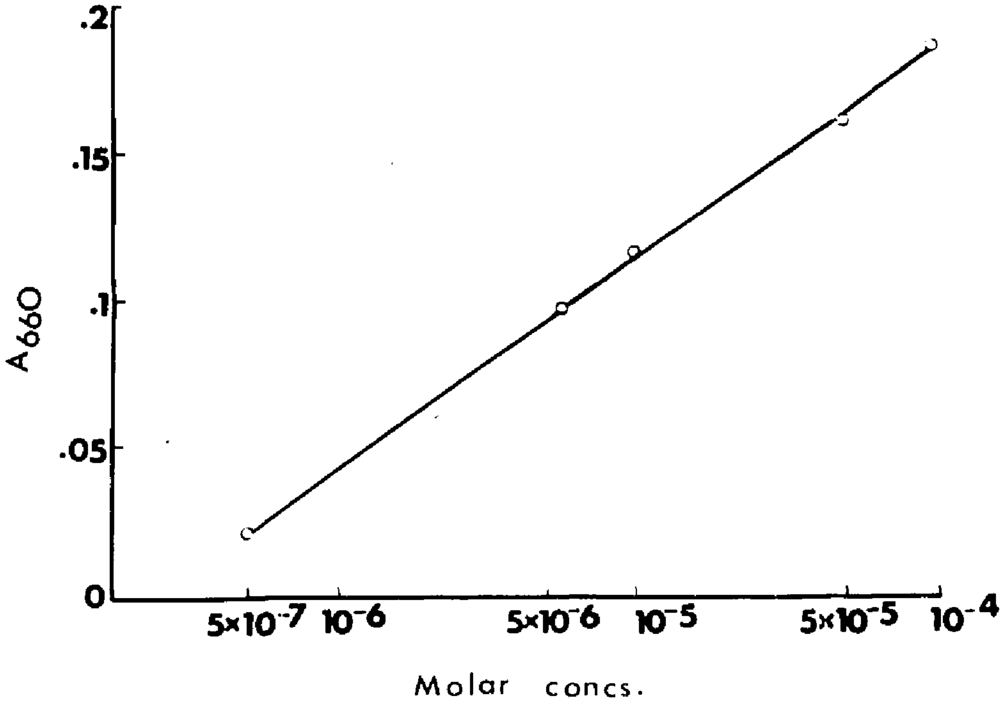


Figure 24. Standard curve for chlorogenic acid.

abscissa = chlorogenic acid (M)

ordinate = $A_{660\ nm}$



Bioassays

The biological activity of IEt was tested using three separate bioassays. In the cucumber radicle test (Fig. 25), IEt proved to be stimulatory to radicle elongation at concentrations below 10^{-6} M. The maximum observable increase in radicle length was at the 5×15^{8} M concentration for both IAA and IEt and the increases were 3.8 mm for IAA and 0.8 mm for IEt (over control). The maximum inhibition was observed at the highest concentration of the growth regulator used (10^{-3} M) and it was 13.4 mm for IAA and 12.0 mm for IEt as compared to controls.

Figure 25. Cucumber radicle inhibition test for indoleacetic acid and indoleethanol.

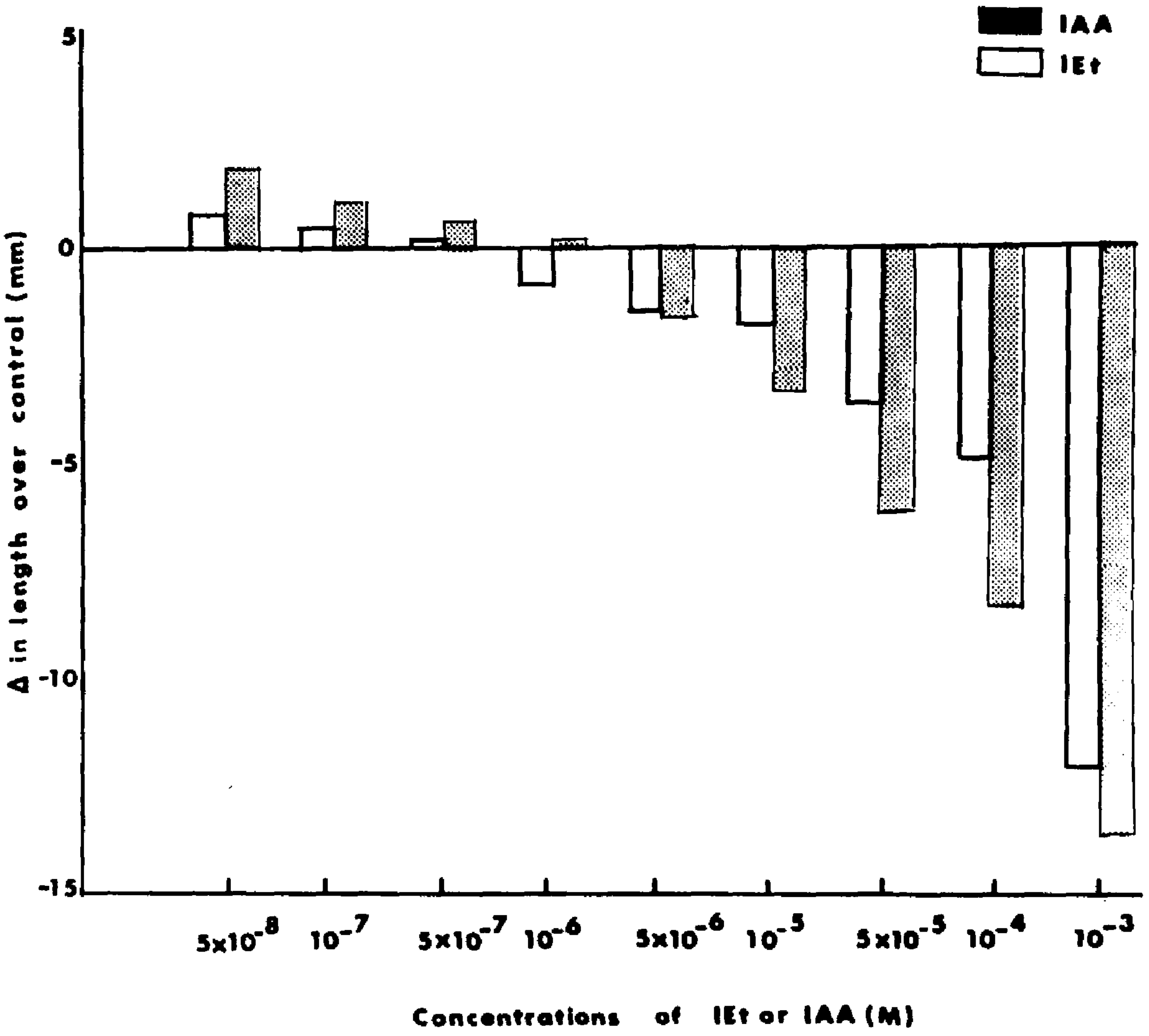
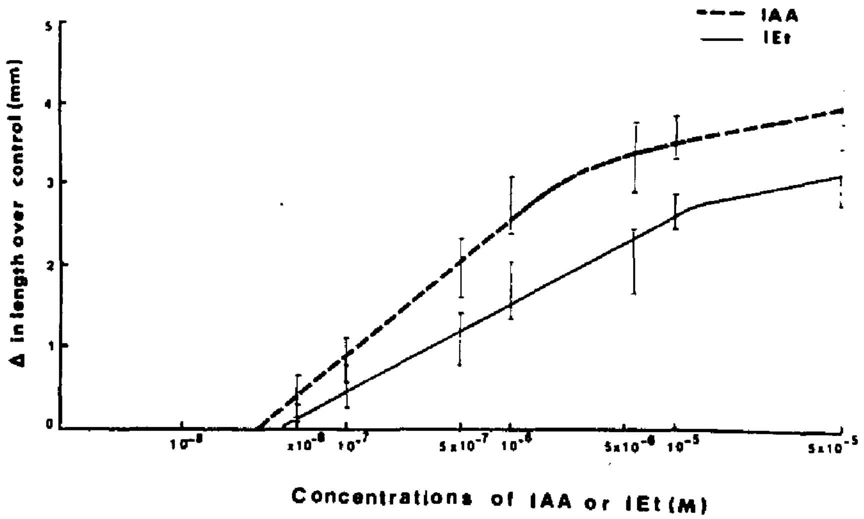


Figure 26. Avena straight growth bioassay for indoleacetic acid and indoleethanol.



In the *Avena* straight-growth bioassay, increases in coleoptile lengths were obtained with concentrations of 10^{-8} M to IAA and concentrations of 5 x 10^{-8} M to IEt (Fig. 26). The magnitude of effect was greater for IAA as compared to IEt for all the concentrations tested. The maximum increase in length occurred at 5 x 10^{-5} M for both IAA and IEt with increases over control of 4 mm and 3 mm, respectively. The results also showed that the linearity of the curve tends to plateau between concentrations of 5 x 10^{-6} M and 5 x 10^{-5} M for both IAA and IEt.

Application of IEt to debladed petioles of Coleus delayed the average days for 50% abscission (Table 15). The three concentrations of IEt applied (10^{-5}, 10^{-4} and 10^{-3} M) delayed abscission (over controls) by 3,8 and 12 days, respectively. Application of 10^{-4} M IAA delayed abscission by 19 days. Use of dimedone (5,5 dimethyl 1,3-cyclohexanedione) along with IEt and IAA did not affect the delaying actions of these substances. Dimedone by itself did not affect significantly rates of abscission.

Table 15. Biological activity of Inodle-3-ethanol: Coleus petiole abscission test.

	Average days for 50% abscission
Control (H_2O)	4
Control and Dimedone	3
IEt (10^{-5} M)	7
IEt (10^{-5} M) + Dimedone	7
IEt (10^{-4} M)	12
IEt (10^{-4} M) + Dimedone	11
IEt (10^{-3} M)	16
IEt (10^{-3} M) + Dimedone	15
IAA (10^{-4} M)	23
IAA (10^{-4} M) + Dimedone	22

Biological activity of extracts

The plants treated with GA_3 were harvested at various days after treatment and the aqueous fraction obtained after partition against butanol was applied to plants. Results showed that extracts obtained from plants two days after treatment with GA_3 caused increased growth of dwarf pea plants (Table 16). Thus, seven days after treatment, while GA_3 treated plants were about 19 cm tall, extract treated plants were about 10 cm tall. Control plants at this stage were about 6 cm tall (Figs. 27, 28). Plants treated with extracts obtained from water-treated controls, were somewhat taller but the difference was not significant.

It was observed that plants treated with GA_3 had greatly extended internodes which was the major reason for the enhanced shoot growth. Results presented in Table 17 show that the second and third internodes had increased dramatically in both GA-treated as well as extract-treated plants. The second and third internode lengths were 7.2 cm and 5.2 cm for GA_3 treated plants and 3.8 cm and 2.4 cm for extract treated plants. The internode number for both control and GA_3 treated plants remained the same (4) while extract treated plants had five internodes.

To check the biological activity of the extracts, the cucumber radicle elongation test was used. It was observed that extracts obtained at various time intervals after treatment with GA_3, inhibited the elongation of cucumber radicles (Table 18). It was observed that the inhibitory effect of the extract diminished when the date of extraction following GA_3 treatment was progressively delayed. So that extracts obtained from plants eight days after GA_3 treatment, only slightly (7.63%) inhibited the elongation of the cucumber radicle. It was noted, however, that extracts from 2AT plants had a more pronounced inhibition (57%) of cucumber radicle elongation than extracts from 1AT plants (42%).

The aqueous fraction obtained after butanol partition (aqueous fraction 1) was further purified by ethyl acetate partitioning (aqueous fraction 2) to avoid any interfering acidic GAs being involved in the growth process. However, results (Table 19, Fig. 29) showed that aqueous extracts obtained after ethyl acetate partitioning was almost as effective as inhibiting radicle elongation as the aqueous fraction 1. The extract obtained from treated plants was subjected to thin-layer chromatography on silica-gel plates and it was observed that two brightly fluorescent areas could be observed under UV-light. These two fluorescent areas were designated as "Upper Rf" and "Lower Rf" and used to test for stem growth and radicle elongation assay. It was observed that the "Lower Rf" would inhibit radicle elongation (by 48%) only.

Table 16. Effect of plant extract on height of dwarf pea plants.

Treatment	Plant height Days after treatment			
	1	3	5	7
Control (H_2O)	2.98	3.65	4.87	5.69
GA_3 (10^{-3} M)	3.90	6.80	13.90	18.52
[1]Extract (2AT)	3.72	6.21	9.44	10.15
[2]Extract (con)	3.11	4.40	5.75	6.43

[1] Extract from plants 2 days after treatment with GA_3.
[2] Extract from plant not treated with GA_3.

Table 17. Effect of plant extract on internode length and number of dwarf pea plants.

Treatment	Length (cm) Internode number 2	3	Number
Control (H_2O)	1.7	1.1	4
GA_3 (10^{-3} M)	7.2	5.2	4
Extract (2AT)	3.8	2.4	5

Table 18. Effect of crude extract obtained from GA-treated plants on cucumber radicle elongation bioassay.

Treatment[a]	Length (mm)	% increase/decrease over control
Control (H_2O)	49.8	- - -
GA_3 (10^{-5} M)	52.0	+4.40
Extract:		
IAT[b]	29.0	-41.76
2AT	21.2	-57.42
2AT[c]	23.0	-53.80
4AT	35.0	-29.71
6AT	39.0	-21.68
8AT	46.0	-7.63

[a] Incubation media

[b] AT stands for days after treatment with GA_3.

[c] Extract was diluted 20-fold in deionized water.

Table 19. Effect of partially purified extract obtained from GA-treated plants on cucumber radicle elongation bioassay.

Treatment[a]	Length (mm)	% increase/decrease over control
Control (H_2O)	50.2	- - -
GA_3 (10^{-5} M)	52.5	+4.58
Aqueous Fraction 1	21.0	-58.16
Aqueous Fraction 2	28.0	-44.22
TLC spot[b]:		
Upper Rf	50.8	+0.01
Lower Rf	26.0	-48.20
Extract from control plants	34.8	-30.67

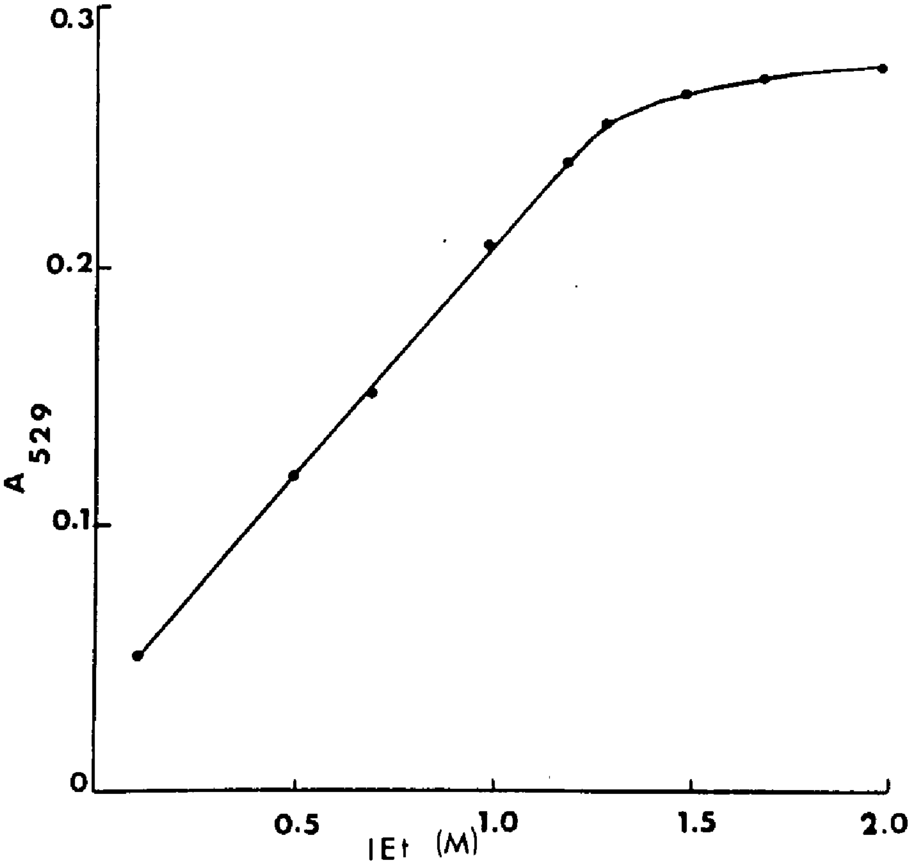
[a] Incubation media.

[b] Extract (2AT) chromatographed and fluorescent Rf areas scraped off.

Figure 27. Saturation curve for the estimation of IEt concentrations in presence of a fixed IAAld concentration using the perchloric acid reagent.

abscissa = IEt concentrations (M x 10^{-4})

ordinate = A_{529}



Discussion

Treatment of stems with gibberellic acid (GA) has long been known to cause increased growth of dwarf pea plants (Brian and Hemming, 1955). Single-gene dwarf mutants of several other species of plants can be induced to normal growth following GA applications (Brian and Hemming, 1955; Phinney, 1956). During the present investigation, application of GA_3 to dwarf pea plants brought about expected increases in plant height. The results also showed that the initial surge of growth occurred about three days after treatment with GA and this was brought about by extension of the second and third internodes. It was also evident from the present investigation that a single application of GA was sufficient for the persistence of this extended shoot growth.

Even though this GA-induced stem elongation has been known for quite some time, there is still some controversy as to the actual mechanism of this process. It has still not been clearly established as to whether the stem elongation is due to cell elongation (Nitsan and Lang, 1966; Rose and Adamson, 1969; Jones and Kaufman, 1971) or by cell division (Bradley and Crane, 1957; Wareing _et al_., 1964; Shininger, 1971) or by both.

There are several reports citing increases in auxin levels of dwarf peas following GA treatments (Kuraishi and Muir, 1964; Valdovinos _et al_., 1967; Law and Hamilton, 1984). The present result thus aimed at elucidating indole metabolism in the pea plant following GA application.

Indoles in dwarf peas

Using five different solvent systems, it was possible to identify several indole substances present in the dwarf pea plant. During the present investigation, plant extracts were taken up in methylene chloride and partitioned at pH 11, 7 and 3 to obtain the basic, neutral and acidic indoles. The first extraction was not carried out at the acidic pH as this has been reported to cause artifactual formation of tryptamine from tryptophan (Liu _et al._, 1978).

The different indoles identified included IAA, IPyA and ILA in the acidic fractions and IAAld and IEt in the neutral fractions. The presence of tryptamine was not detected in any of the experiments. It was observed during the present investigation that use of Silica gel HL plates (pre-heated at 60°C for 1 hr) proved to be the most satisfactory solid phase when used in the toluene: ethyl acetate: acetic acid solvent system. This system was particularly well-suited to the separation of IEt, IAA and IAAld.

Auxins have been detected in extracts from pea plants using biological assay techniques, and are at higher concentrations in shoots than in roots (Cartwright _et al._, 1956). Evidence for the presence of IAA itself came from earlier work on bioassays of zones on chromatograms corresponding in R_f with the known compound (e.g. Kuraishi and Muir, 1964), along with visualization of chromatogram spots by indole reagents (Bennet-Clark and Kefford, 1953; Rajagopal, 1967). Recently, Law and Hamilton (1984) have shown its presence by an isotope dilution HPLC method. The presence of IAAld in the pea plant was first suggested by Rajagopal (1967) based on chromatography, bioassay, color reactions and UV spectra and was later confirmed in sterile tissue (Rajagopal, 1968a).

IEt has long been suggested as an intermediate formed during the conversion of TPP to IAA (Wightman, 1964). Its presence had been frequently cited in indole metabolism studies of bacteria and fungal species (Kaper and Veldstra, 1958; Bailey and Gentile, 1962; Larsen *et al.*, 1962; Perley and Stowe, 1966). Also, Rajagopal (1967) found that all the 33 plant species (from 17 families, but not dwarf peas or other dwarf varieties) tested could metabolize IAAld to IEt. The natural occurrence of IEt was further demonstrated in cucumber seedlings using mass spectrometry (Rayle and Purves, 1967) and in pine needles by combined gas chromatography - mass spectrometry (Sandberg, 1984). Marumo *et al.* (1968a,b) have reported the presence of a chlorinated auxin, methyl-4-chloroindol-3yl-acetic acid in immature pea seeds. Recently, Wightman and Schneider (1985) have reported the presence of 3-indole propionic acid, 3-indolebutyric acid and phenyl acetic acid in pea seedlings by using HPLC and GC-MS procedures.

The effect of GA_3 application on auxin levels of dwarf pea plants was estimated during the present investigation. The results showed increased IAA production in GA_3 treated plants (Fig. 2). It was observed that even though IAA levels were highest three days after GA_3 application, the treated plants had increased IAA levels all through the observation period.

The amount of IAA present in the shoots of dwarf pea plants varied from 50 to 81 ng/g fresh weight. While there have been many reports of quantitative estimations of auxin level in plants, the differences in extraction and assay methods make comparisons difficult.

Results vary from 5 ng/g fr wt in <u>Triticum vulgare</u> leaves (Shaw and Hawkins, 1958) to 100 ng/g fr wt in <u>Nicotiana longiflora</u> stem tissue (Bayer and Ahuja, 1968). Schneider <u>et al</u>. (1972) have compared some of the older works and have suggested that variations apparent in these results may also be due to such factors as age of plant when determinations were made and the variety tested.

Ever since Nitsch (1957) reported increased extractable auxin levels in <u>Rhus</u> plants following GA_3 application, there have been numerous similar observations in a wide variety of plants. Thus, dwarf and tall peas (Phillips <u>et al</u>., 1959; Kogl and Elema , 1960; Kuraishi and Muir, 1962; 1963), sunflower (Kuraishi and Muir, 1962), <u>Parthenocissus</u> tissue culture (Butenko <u>et al</u>., 1961), rice (Yerygin <u>et al</u>., 1961), potato (Reingard and Palladina, 1963), cabbage and corn (Radionova and Runkova, 1963) and celery (Kato and Ito, 1962) have all been shown to have increased auxin levels following gibberellin treatments. In Little Marvel dwarf pea seedlings, Valdovinos <u>et al</u>. (1967) reported a two-fold increase in the diffusible auxin level of GA_3-treated plants. Law and Hamilton (1984) were able to detect three times more IAA in lower stem segments of GA_3-treated Little Marvel peas. In the present investigation, the close to two-fold increase in the IAA level of the GA-treated plants agrees with the above results.

<u>Radiolabelled precursor study</u>

Assays of tryptophan-3-^{14}C conversion by cell free preparations of the apical tissue revealed that tryptophan metabolism was increased by GA_3 treatment. Increased TPP conversion proceeded with increase in incubation time. The major radioactivity readings were found to be associated with the acidic and neutral indole fractions. TLC scans of the acidic and neutral fractions revealed two major peaks corresponding to IAAld and IEt for the neutral fraction and one major peak corresponding to IAA for the acidic fraction.

These results suggest that TPP is converted to IAA in the dwarf pea tissue via conversion to IAAld.

Since Thimann's studies showing that cultures of *Rhizopus suinus* could produce IAA from TPP (Thimann, 1935), a number of studies (both in vivo and in vitro) have been carried out to elucidate the enzymatic conversion of TPP to IAA (Wildman *et al.*, 1947; Gordon and Sanchez, 1949; Libbert, 1962; Lantican and Muir, 1969).

Tracer studies have shown that radioactivity from exogenously supplied (^{14}C- or ^{3}H)-labelled TPP was incorporated into IAA. This was demonstrated with watermelon tissue slices (Dannenburg and Liverman, 1957), in *Lens* roots and epicotyls (Pilet, 1961), cabbage shoots (Wightman, 1962), lime fruit (Khalifah, 1967), maize endosperm (Hall and Bandurski, 1978) and tobacco petiole tissue (Liu et al., 1978). Several investigators have reported ^{14}C TPP conversion to IAA in the pea plant (Kutacek and Kefeli, 1970; Moore and Shaner, 1967,1968; Moore, 1969; Valdovinos *et al.*, 1967).

Experiments in which ^{14}C-labelled indole and ^{3}H-serine or (^{14}C, ^{3}H) double-labelled tryptophan was fed to tips of pea seedlings have shown the ^{14}C:^{3}H ratios of the supplied precursor and of IAA produced to be the same (Erdmann and Schiewer, 1971). These results suggest that no bypass from indole to IAA exists in this plant material. Such results have also been shown in maize (Heerkloss and Libbert, 1976 a,b).

Pathway of IAA formation from TPP

The conversion of tryptophan into IAA involves a side-chain degradation. The exact pathway, however, still remains controversial.

During the present studies, D,L-tryptophan-3-^{14}C incubation with a cell free preparation of pea stem tissue did not show any detectable radioactivity which could be associated with IPyA. However, the detection of IPyA is difficult because of the small amounts formed and its instability during extraction and chromatography (Gibson et al., 1973). On the other hand, there was no evidence of any tryptamine formed by decarboxylation of TPP. The presence of IPyA was, however, detectable when the acidic fraction of plant extracts were chromatographed on cellulose TLC plates with the BAW or TEA solvent system (Table 1).

The presence of IAAld, the immediate precursor of IAA, was detected in the radiochromatograms as well as in TLC of neutral fractions of plant extracts (Fig. 4). The formation of IAAld from TPP has been shown by various workers also. Khalifa (1967) detected the presence of a neutral, radioactive compound after feeding TPP-3-^{14}C to lime fruits and concluded this to be IAAld based on its Rf value in silica-gel column chromatography. Similar results were also obtained by Phelps and Sequeira (1968) in tobacco and Gibson et al. (1972) in mung bean tissue homogenate. The presence of IAA as the product of TPP metabolism was concluded on the basis of radioactivity scan of the TLC plate of the acidic fraction (Fig. 3). It was seen that the major peak of radioactivity coincided with the Rf of authentic IAA. The formation of IAA was also confirmed by extraction of the acidic fraction, TLC or TEA solvent system and finally the indole α-pyrone assay.

The present investigation also showed that incubation of a cell free preparation with TPP led to the formation of significant amounts of the neutral indole, IEt. The radioactivity peak (around Rf 0.3) in the neutral zone corresponded well with authentic IEt which had an Rf of 0.29 in the same system. The IEt was also detected in TLC plates by spraying with Ehrlich reagent and the Rf value compared with known IEt. The Rf zone corresponding to IEt was also identified under UV, scraped off the plate and allowed to react with the perchloric Salkowski reagent. This yielded a characteristic pink color that had an absorption maxima at 529 nm (Brown and Purves, 1976).

On the basis of the above results, it is concluded that in the dwarf pea plants IAA biosynthesis takes place according to the indolepyruvic acid pathway and involves the formation of indolepyruvic acid and indoleacetaldehyde as intermediates (Appendix I). The IAAld could, however, also be diverted to form IEt - the significance of which has never been established.

Results show that GA_3 treatment causes significant increases in the IAA present in the dwarf pea plants. It is inferred that this IAA is mostly being synthesized as evidenced from the large increase in the IAAld level following TPP-3-^{14}C incubation (Fig. 4). It is also noted that along with this increase in the level of acidic indoles, GA_3 treatment also causes significant increase in the amounts of neutral indoles present. One of the reasons for this being the diversion of IAAld to form IEt. Kuraishi and Muir (1964) found that enzyme breis from GA-treated dwarf pea seedlings converted approximately four times more "water soluble auxin" than enzyme breis from water-treated (control) seedlings. The fraction referred to as "water soluble auxin" by Kuraishi and Muir is that auxin which remained after ether-extraction at an acidic pH. This fraction would include the neutral indoles (Powell, 1964). The production of IEt by tissues provided with IAAld has been reported in bacteria, fungi and a wide variety of plants (Rajagopal, 1967; Gibson _et al_., 1972; Sandberg, 1984). Gibson _et al_., (1972) observed that tomato plants fed with D,L-TPP-3-^{14}C showed a five-fold increase in the amount of IEt formed. In pine needles, IEt was shown to be produced from TPP-3-^{14}C and 2-^{14}C tryptamine by an enzymic reaction (Sandberg, 1984).

Indolepyruvic acid pathway enzymes

After establishing the pathway for IAA biosynthesis from TPP in the dwarf pea, it was of interest to investigate the enzymes involved in the pathway and assess the possible role of GA_3 in altering their activities.

Tryptophan aminotransferase:

Transamination of TPP to form IPyA is the first step and is catalyzed by a widely distributed multispecific aminotransferase (Wightman and Cohen, 1968; Truelsen, 1972; Matheron and Moore, 1973). The enol-borate buffer assay of Lin _et al_. (1958) was employed to assay the yield of IPyA formed during the reaction. The indolepyruvate-borate complex has a maximum absorption at 328 nm and standard curve prepared from known amounts of IPyA showed that it was linear.

The aminotransferase was purified about 82 fold by acetone precipitation followed by Sephadex G-200 column chromatography (Table 4). The optimum pH and temperature for the catalytic conversion were about

8.5 and 40°C respectively. Using D,L-TPP as a substrate the K_m value was found to be 4×10^{-4} M (Fig. 7). The enzyme was able to convert tryptophan, phenylalanine, leucine and alanine as substrates. Pyruvic acid and oxalacetic acid were as effective as α -ketoglutaric acid as amino group acceptors. The stoichiometric formation of indolepyruvate and glutamate (1.18 and 1.20 u moles respectively, with D,L-tryptophan) showed that IPyA was being formed by transamination and not by some other process.

The effect of GA_3 treatment on TPP transaminase was also investigated (Fig. 8). It was observed that the specific activity of the enzyme was increased following GA_3 treatment. The maximum increase in activity of the enzyme occurred by the third day of treatment which also coincided well with increased IAA level at this stage (Fig. 2). The fact that the pea aminotransferase was more active with D-TPP than with L-TPP appears unusual (Table 5). Most reports suggest the specificity of the enzyme for the L-form only (Wightman and Cohen, 1968; Gibson _et al._, 1972; Truelsen, 1972). However, there are several reports of D-TPP being converted by plant enzymes (Gordon, 1961; Gamborg and Wetter, 1963; Matheron and Moore, 1973). In fact, Gordon (1961) reported that D-TPP was equal to or more effective than L-TPP as a precursor of IAA in several plant preparations. Matheron and Moore (1973) reported that the D-form was three times more effective than the L-form in pea TPP transamination. Also, in experiments where TPP has been found to mimic the effects of IAA, the D- and L-forms of TPP have been found to be equally effective (Valdovinos and Perley, 1966). Valdovinos and Perley (1966) found that various concentrations of D- and L-TPP were equally effective in delaying abscission of debladed Coleus petioles. The conversion of D-TPP to malonyl-D-TPP has been shown to occur enzymatically in many plant species (Good and Andrae, 1957; Miura and Mills, 1971).

IPyA decarboxylase

An enzyme decarboxylating IPyA to IAAld was observed to be present in pea tissue homogenates. The difficulty in working with IPyA was circumvented by utilizing the borate buffer system to stabilize the IPyA. The enzyme reaction was calculated after exterpolating from blank determinations the chemical conversion of the acid. Also, it was observed that by stopping the reaction with a bisulfite, the further conversion of IAAld to IEt or IAA was prevented. This was found to give a more accurate status of the decarboxylase enzyme as compared to measurement of the final product (e.g. IEt) as reported earlier (Wightman, 1973; Liu *et al.*, 1978).

The effect of GA_3 on IPyA decarboxylase activity was measured at several stages after treatment but was not found to be significantly affected (Table 7). However, even though the present investigation employed a modified procedure for the estimation of IPyA decarboxylase activity, it is possible that the apparent labile nature of the substrate made it difficult to run this assay. Readings may therefore be modified by different rates of IPyA breakdown of which there are few if any reports.

IAAld dehydrogenase

The enzymatic conversion of IAAld to IAA has been shown to occur in plant tissue and tissue extracts by various workers (Larsen, 1944, 1951; Clarke and Mann, 1957; Gordon and Sanchez, 1949; Phelps and Sequeira, 1967; Rajagopal, 1967, 1968a,b; Wightman and Cohen, 1968; Gibson *et al.*, 1972).

An NAD-dependent IAAld dehydrogenase was present in crude enzyme brei capable of converting IAAld to IAA. This was purified by passing a 50% $(NH_4)_2 \cdot SO_4$ fractionation through a Sephadex G-100 column (Table 8). The major peak of activity was obtained with one protein having a M.W. of 22,500 and two smaller protein peaks (M.W. 9,200 and 40,000). The pH maxima of the enzyme was at 5.5 (Fig. 11). The IAAld dehydrogenase enzyme was found to be affected by the presence of dimedone in the reaction mixture (Table 9). These results demonstrated that dimedone can tie up IAAld and prevent its conversion to IAA.

Treatment of plants with GA_3 was observed to affect the specific activity of IAAld dehydrogenase one day following treatment (Fig. 12). Thus, the activity of the enzyme, though higher at days 4 and 5 (over controls), followed the pattern of untreated plants over the observation period.

An NAD-dependent IAAld dehydrogenase has been shown to be present in the cytoplasmic supernatant preparations of mung bean seedlings (Wightman and Cohen, 1968) and tomato shoots (Wightman, 1973). On the other hand, an NAD-independent oxidase has been shown to be present in several species like *Avena* coleoptiles (Rajagopal, 1971), tobacco callus tissue (Liu *et al.*, 1978) and cucumber seedlings (Bower *et al.*, 1976).

IAAld reductase

Larsen (1951) presupposed the formation of IEt from IAAld on the basis of his observation that for each mole of IAA produced by Avena tissue, two moles of IAAld disappeared. Rajagopal (1967) subsequently observed that all the 33 plant species tested would metabolize IAAld to IEt.

A reductase enzyme was partially purified that would convert IAAld to IEt from pea tissue homogenate. Sephadex chromatography results show basically a single protein (M.W. 40,000) which is NAD(P)H dependent and has a pH optima of about 7.0. This is quite similar to the IAAld reductase separated from cucumber seedlings having a M.W. of about 32,000 (Brown and Purves, 1980). However, the enzyme obtained from cucumber seedlings had differential behavior when NADH or NADPH was used (Brown and Purves, 1980). In the present investigation, no such difference was observed with either NADPH or NADH.

The reaction rate of the IAAld reductase in presence of NADPH shows typical Lineweaver-Burk kinetics (Fig. 17). A K_m value of 208 uM for IAAld was obtained under these conditions. This is comparable to the K_m value (125 uM for IAAld) of the enzyme obtained from cucumber seedlings (Brown and Purves, 1980). However, the cucumber enzyme showed a strong dependence of the kinetic parameters on pH and ionic strength. Thus, without actual knowledge of intracellular pH and ionic strength, no valid assumption of substrate affinity of this enzyme can be made.

The pea IAAld reductase was also found to be affected by the presence of IAA in the reaction mixture (Table 12). It was observed that

50 uM of IAA would cause about 50% inhibition of the enzyme activity. Attempts to elucidate the nature of this inhibition proved unsatisfactory. This does not support the concept of the role of IEt being confined to that of a storage form which would divert away from excessive IAA production (Brown and Purves, 1980; Sembdner *et al.*, 1980). Bower *et al.* (1976) showed that the auxin analog 2,4-D inhibited oxidation of IEt to IAAld. On the basis of this result, Brown and Purves (1980) concluded that this was a feedback regulatory mechanism to control IAA synthesis. During the present investigation, no oxidase for conversion of IEt to IAA could be detected in the dwarf pea plants. Moreover, high concentrations of IAA were found to inhibit IEt production which is contrary to a notion of feedback inhibition. The present results on IAAld reductase activity while substantiating the role of IEt as being important in indole metabolism (Rajagopal, 1967; Brown and Purves, 1976), suggests IEt to have a more direct role in plant growth than has previously been considered.

The treatment of plants with GA_3 was observed to affect IAAld reductase activity of dwarf pea plants. Results suggest that treated plants had a higher activity of the enzyme as compared to controls.

Oxidation of IAA

During the present investigation an attempt was made to observe the rate of IAA oxidation in the dwarf peas and determine whether this was a basis for increased IAA levels following GA_3 treatment. Results (Figs. 19, 20 and 21) suggest that use of a synthetic substrate like o-dianisidine may not give an accurate picture of the IAA-oxidising system of plant tissues. The protein profiles responsible for the o-dianisidine color reactions did not match exactly when the loss of IAA was measured with IAA as a substrate (Figs. 19 and 20). The present results also show very little difference in IAA oxidation levels of control and GA_3-treated plants. Reports of the effects of GA_3 on IAA oxidase and peroxidase levels are conflicting. Galston and McCune (1961) reported decreased level of the oxidation system in peas and corn following GA_3 treatment. Similar results were obtained with crude extracts which, however, showed no differences when the enzymes were purified (Kogl and Elema, 1960). Pilet (1961) reported that an inhibitory effect of GA_3 on IAA oxidation in excised Lens root tissue in fact became promotive under in vivo conditions. Several reports also report no effect of GA_3 on the purified enzyme in vitro (e.g. Watanabe and Stutz, 1960; Kogl and Elema, 1960; Brian and Hemming, 1960).

The phenols have been considered to affect the rates of IAA oxidation (Mudd and Burris, 1959; Sacher, 1963; Gelinas and Postlethwait, 1969). While p-monophenolic compounds increase the rate of IAA degradation, the 3,4-disubstituted phenols are generally inhibitory (e.g. Grambow and Langenbeck-Schwich, 1983). It was observed during this investigation that the presence of a disubstituted phenol (caffeic acid) delayed the rate of IAA oxidation (Fig. 22). The results also suggest that this delay is caused by the increased lag period to IAA oxidation in the presence of caffeic acid. This could possibly be due to an inactive complex formation with the oxidative enzyme (Gelinas, 1972) or a steady reductive regeneration of IAA from an oxidized but not decarboxylated species (Grambow and Langenbeck-Schwich, 1983). The present results also suggest the difference in action of the different fractions. Thus, while caffeic acid increased the lag period of one enzyme fraction, it did not have any effect on the other (Table 14). Such a result suggests a possible difference in the mechanism of action of the IAA oxidase/peroxidase system.

Phenols

The effect of GA_3 on the total ethanol-soluble phenol level was observed to be affected at early stages following treatment. There was a significant increase in the total phenol level three days following GA_3 treatment. Although individual phenolic substances were not isolated, TLC scan of the different phenols shows the presence of an unidentified band present in GA_3 treated plant extracts (Fig. 23). Kogl and Elema (1960) reported increased amounts of polyhydroxycinnamic acids (Ferulic, caffeic and chlorogenic acids) after GA_3 treatment. However, Sagi and Garey (1961) found no effect of GA_3 on the phenol content in leaves of treated plants. Gelinas and Postlethwaite (1969) were able to relate the levels of a ferulic acid derivative with mutants of maize. Fry (1979) hypothesized that the mode of action of GA_3 could be mediated through its effect on esterified ferulic and levels of plant cells which apparently have an important role in rigidifying cell walls.

Role of IEt in plants

The growth promoting activity of IEt has been demonstrated with different plant material (Larsen, 1944; Purves _et al._, 1967; Rayle and

Purves, 1968; Evans and Rayle, 1970). Rayle and Purves (1967) observed that IEt was nearly as effective as IAA in the cucumber hypocotyl assay. They also reported it to be a strong growth promoter in several other species. Other reports have also suggested biological activity of this indole (Veldstra, 1953; Wightman, 1962).

It has been suggested that the biological activity of IEt is mediated through its conversion to IAA via IAAld (Gibson et al., 1972; Vickery and Purves, 1973). In this investigation, repeated attempts failed to show the activity of an oxidase for converting IEt to IAAld in the dwarf pea plants such as described in cucumber by Vickery and Purves (1972).

Valdovinos and Perley (1966) used debladed Coleus petioles as a test system for measuring the activity of auxin and precursors. They confirmed that application of IAA would significantly delay the days for 50% abscission of the petioles. They also found that while application of tryptophan and tryptamine would delay the abscission, use of certain inhibitors would negate this effect. Thus, dimedone, which acts as an aldehyde trap (Clark and Mann, 1957) and Parnate, an inhibitor of monoamine oxidase (Perley and Stowe, 1966), when applied together with tryptophan and tryptamine, removed the delaying effects of these substances on the abscission of petioles. It was observed in the present investigation that dimedone had no effect on the days for 50% abscission of either IEt or IAA treated petioles. It appears unlikely that IEt was acting on abscission as a precursor for IAA. The Avena straight growth test showed a loss of linearity in the curve beyond the 10^{-5} M concentrations for both IAA and IEt. If IEt was acting as a precursor for IAA, it is unlikely that a plateau in growth would have been reached in the 10^{-5} M concentration range. The concentration range for activity of IEt in the Avena test suggests that its action is more direct than as a precursor for IAA. The effects of IEt on cucumber radicle elongation were quite similar to IAA in the same concentration range. The evidence suggests a more direct role for IEt than as an auxin precursor.

The presence of phenylacetic acid and several indoles (including 3-indolebutyric acid and 3-indolepyruvic acid) have been established in plant tissues in recent times (Milborrow et al., 1975; Segal and Wightman, 1982; Wightman and Schneider, 1985). However, little is known about the possible regulatory roles of these substances in plant growth and development. Without adequate knowledge of site(s) of action, transport or possible intracellular localization, it is difficult to conclude which substances are actively involved in growth regulation. Therefore, it seemed desirable to re-evaluate the role(s) of the indole metabolite IEt. The results of the present investigation indicate that IEt may be more directly involved in plant growth than as a precursor for IAA.

Finally, an attempt was made to elucidate the mechanism of GA_3-induced increase in height of dwarf pea plants. Numerous theories have been postulated over the years to explain this phenomenon. These include the concept that dwarf plants are incapable of converting 3β-hydroxylation of GA_{20} to GA_1 which is responsible for internode elongation (Potts et al., 1982; Potts and Reid, 1983), inhibition of growth by higher level of phenolic inhibitor (Chailakhyan, 1979), greater cell metabolism through changes in activities of enzymes (Broughton and McComb, 1971) and prevention of IAA oxidation through accumulation of phenolic substances (Fry, 1979). None of the above hypotheses have demonstrated conclusivelv the exact mechanism of GA-induced stem elongation in the dwarf pea plant.

During the present investigation an attempt was made to investigate the possible presence of a substance/s that may be present in plants treated with GA_3. It was observed that an aqueous fraction obtained after butanol-partioning at neutral pH, when applied to plants, would induce stem elongation of treated plants (Table 16). Studies showed that an active substance could be obtained from GA_3-treated plants even when extractions were made eight days after treatment. The "extract" treated plants had increased internode elongation but also had more internodes (Table 17). Also, these plants had broader leaves and stipules and did not look morphologically the same as GA_3-treated plants.

In order to rule out possible effect of residual exogenous GA_3, the aqueous fraction was further subjected to ethyl acetate extraction at pH 3 to remove growth-promoting GAs. The aqueous fraction remaining was again applied and found to produce the same activity. When the "extracts" were subjected to the cucumber radicle bioassay, it was observed that drastic inhibition of root growth took place (Tables 18 and 19). The present results suggest that foliar application of GA_3 triggers the formation of a growth-promoting substance that causes elongation of dwarf pea stems. That this substance is not a biologically active GA can be concluded on the following basis:

i) it was obtained in a neutral fraction after the ethyl acetate extraction of acidic GAs (most free GAs).

ii) it inhibited cucumber root growth whereas authentic GA_3 had no effect.

iii) extracts made two days after treatment proved more active than those made one day after treatment.

iv) extracts from control (H_2O treated) plants had a slight effect on shoot elongation but a more dramatic effect on root inhibition.

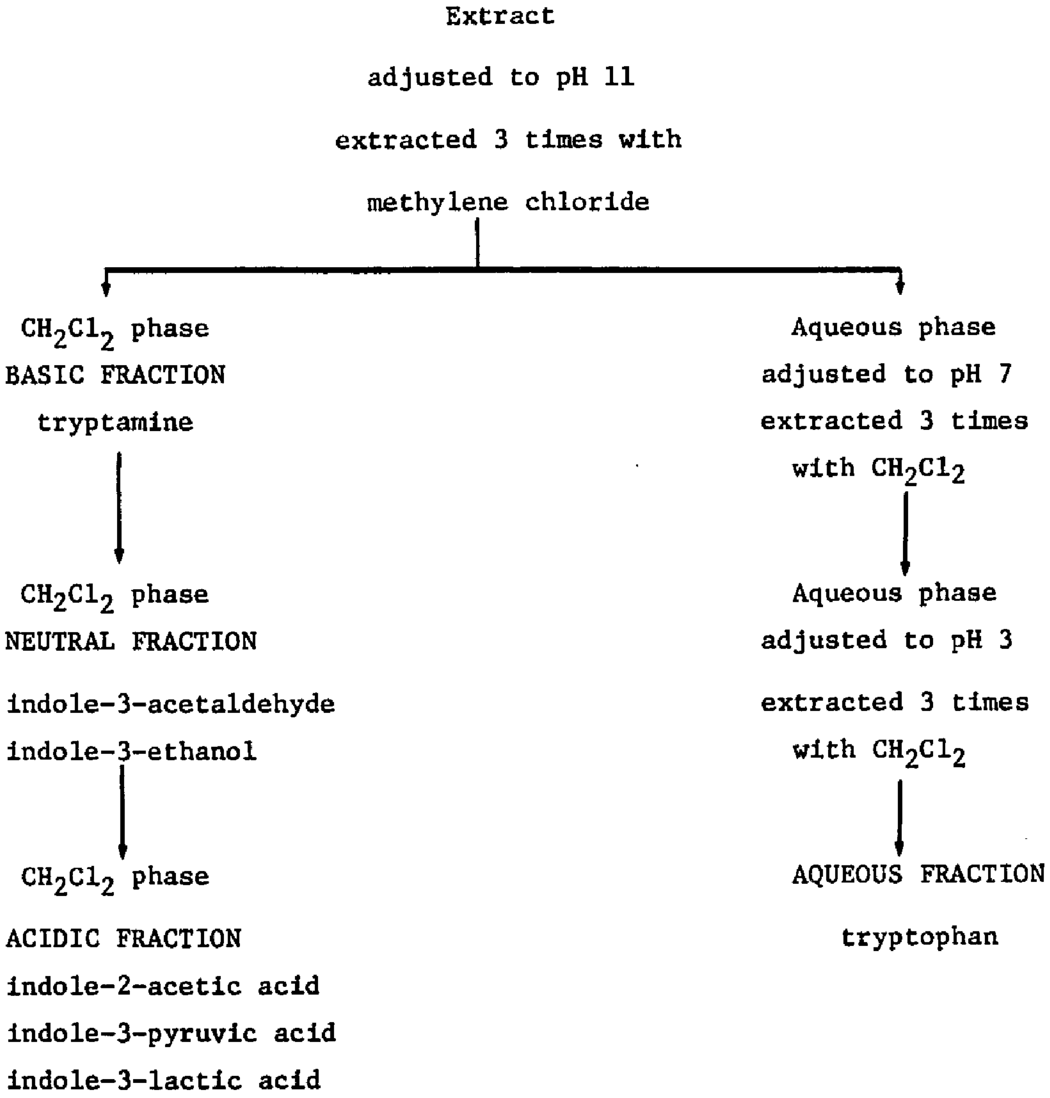
Even though recent evidence on the biochemical basis of dwarfism has suggested that the lack of 3 β -hydroxylation of GA_{20} to GA_1, as being the cause of the dwarf phenotype (Potts and Reid, 1983; Reid _et al_, 1983), there still remain some unexplained questions. Thus, it cannot be explained as to how dwarf plants can still respond (even though weakly) to GA_{20} applications if the _Le_ gene is responsible for 3 β -hydroxylation. Moreover, Gaskin _et al_. (1985) showed that there was very little difference in the different GAs present in developing seeds of Alaska (tall) and Progress No. 9 (dwarf) peas. They could also detect the presence of equal amounts of GA_1 in the dark grown tall and dwarf plants. McComb and McComb (1970) reported that the effect of the _Le_ locus is not graft transmissable. They concluded that the block to rapid growth in plants homozygous for _le_ was controlled not by gibberellin synthesis itself but by the "events linking gibberellin with the growth response." The present results suggest a similar conclusion. It is suggested that the effect of GA_3 is mediated by the production of a substance/s that is responsible for causing elongation of plants. This would also account for the lack of activity of GA on pea stem segments where the "active" substance probably cannot be synthesized.

The exact chemical nature of this growth substance is being further investigated.

APPENDICES

Appendix A

Fractionation of indole compounds.



Appendix B

Ehrlich reagent

84 ml of 95% ethanol

16 ml of conc. HCl

2 g of p-dimethylaminobenzaldehyde

Salkowski reagent

100 ml of 70% perchloric acid

4 ml of 0.5 M $FeCl_3$ solution

Added to 100 ml of distilled water

Appendix C

For tryptophan-3-^{14}C incubation studies D,L-TTP-3-^{14}C (specific activity of 3.716 mc/mm) was used.

Incubation medium contained:

0.154 mg cold TTP
0.25 ml TTP-3-^{14}C
5 ml enzyme solution
4.5 ml water
0.5 ml chloramphenicol (0.25 mM)

Counting solution:

95 ml Liquifluor
276 ml Toluene
329 ml Triton X-100

Countings taken with Nuclear-Chicago 725 series liquid scintillation counter.

Appendix D

Buffers:

Phosphate buffer, pH 5.9, 0.05M

0.1 M K_2HPO_4	10 ml
0.1 M KH_2PO_4	90 ml
distilled water	100 ml

Phosphate buffer, pH 7.0, 0.1 M

0.2 M K_2HPO_4	61 ml
0.2 M KH_2PO_4	39 ml
distilled water	100 ml

Phosphate buffer, pH 8.0, 0.1 M

0.2 M Na_2HPO_4	94.7 ml
0.2 M NaH_2PO_4	5.3 ml
distilled water	100 ml

Boric acid - borate buffer, pH 8.5

0.2 M boric acid	50 ml
0.05 M borax (0.2 M sodium borate)	13 ml
distilled water	100 ml

Tris-HCl buffer, pH 7.2 to 7.5

I = 0.2 M tris (hydroxymethyl) aminomethane

II = 0.2 M HCl

a) pH 7.2: 50 ml of I and 41.2 ml of II

b) pH 7.4: 50 ml of I and 41.4 ml of II

c) pH 7.5: 50 ml of I and 40 ml of II

All solutions made up to 200 ml with distilled water.

Sodium acetate buffer, pH 5.0

0.2 M acetic acid solution (11.55 ml in 1000 ml)	14.8 ml
0.2 M sodium acetate	35.2 ml
distilled water	100 ml

Citrate buffer, pH 5.0

0.1 M citric acid	20.5 ml
0.1 M sodium citrate	29.5 ml
distilled water	100 ml

Appendix E

Ninhydrin reagent for detection of glutamic acid on thin-layer plates (Waldi, 1965).

0.3 g Ninhydrin was dissolved in 100 ml of n-butanol and mixed with 3 ml of glacial acetic acid.

Appendix F

Column chromatography

Column dimensions = 2.5 x 45 cm for G-25 and G-100
1.5 x 35 cm for G-200

Bed height = 30 cm (G-25);35 cm (G-100);25 cm (G-200)

Bed volume - 125 ml (G-25); 235 ml (G-100); 160 ml (G-200)

Flow rate = 9 drops/min (G-100); 7 drops/min (G-200); 25 drops/min (G-25).

Operating pressure - 30-35 cm (G-100); 13-16 cm (G-200)

Void volume was determined with blue dextran 2000 and was found to be about 35 ml for G-100 and 19 ml for G-200.

Gel swelling: 15 g of Sephadex G-100 dry powder were swelled in 300 ml phosphate buffer, pH 5.9 plus 0.03 M NaN_3 for 5 hr in boiling water bath.

Column packing: After the column had been levelled, the preswollen gels were poured with a long glass stirring rod. Flow adaptor, 4 way valve and Marriott flask were attached to the column and the entire assembly was kept at about 4°C by circulating cold water by means of a water circulator (Forma Scientific Regrigerated Circulator). Fractions were eluted with 0.05 M potassium phosphate buffer, pH 5.9.

Partition co-efficient:

$K_{av} = (V_e - V_o)/(V_a - V_o)$

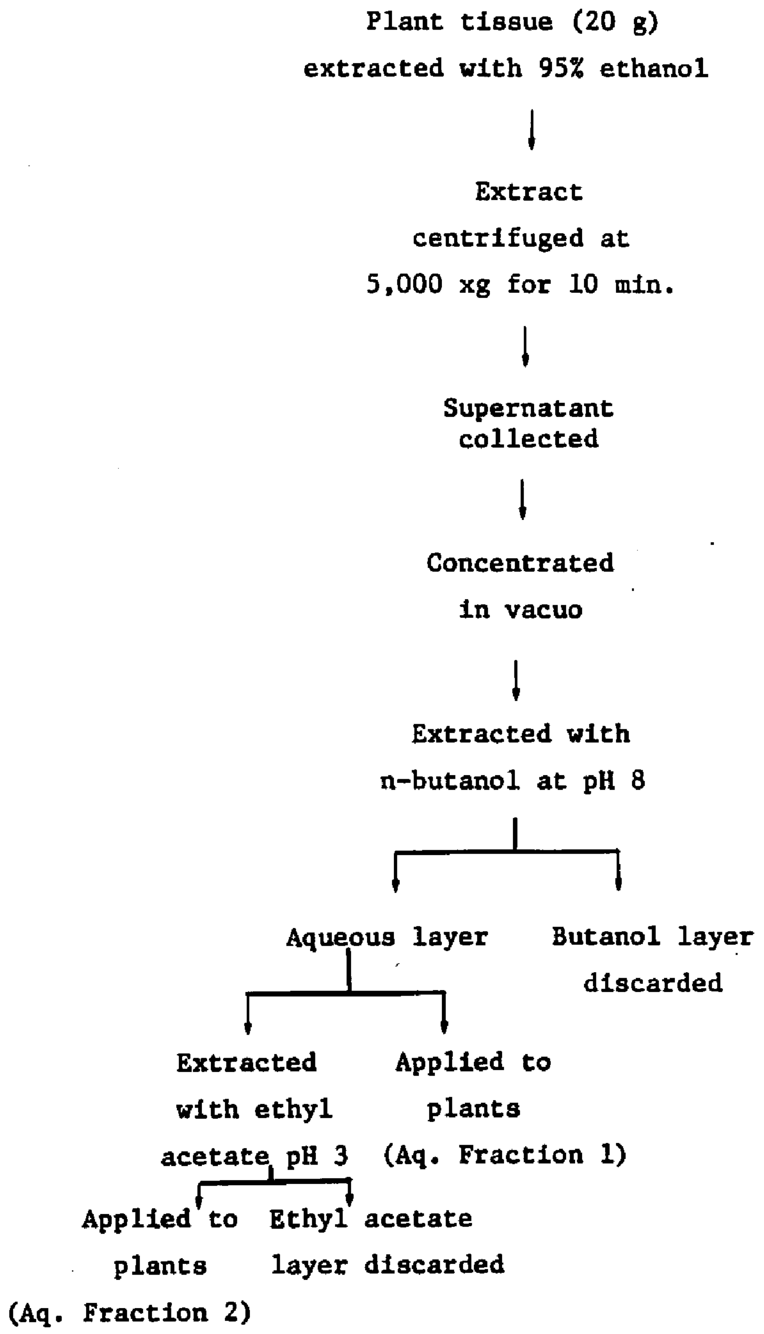
V_e = elution volume

V_o = void volume

V_a = bed volume

Appendix G

Extraction of growth-promoting substance



Appendix H

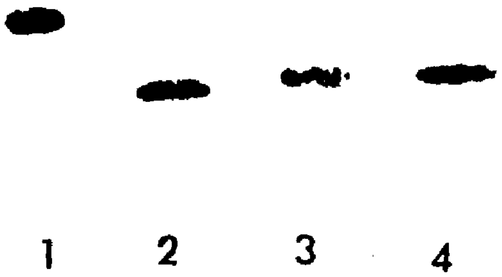
Production of IEt from IAAld by IAAld reductase enzyme.

1 = IAA (reference spot)

2 = IEt (reference spot)

3 - IEt formed after 15 min. incubation

4 = IEt formed after 1 hr. incubation



Appendix I

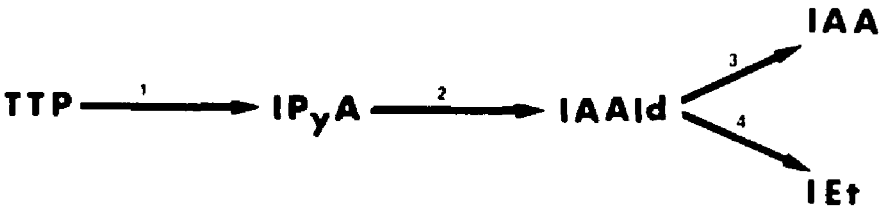
The IAA biosynthetic pathway in the dwarf pea plant.

1 = TPP aminotransferase

2 = IPyA decarboxylase

3 = IAAld dehydrogenase

4 = IAAld reductase



Appendix J

Effect of "plant extract" on growth of dwarf pea plants.

A = Control (H_2O treated)

B = Extract treated

C = GA_3 treated

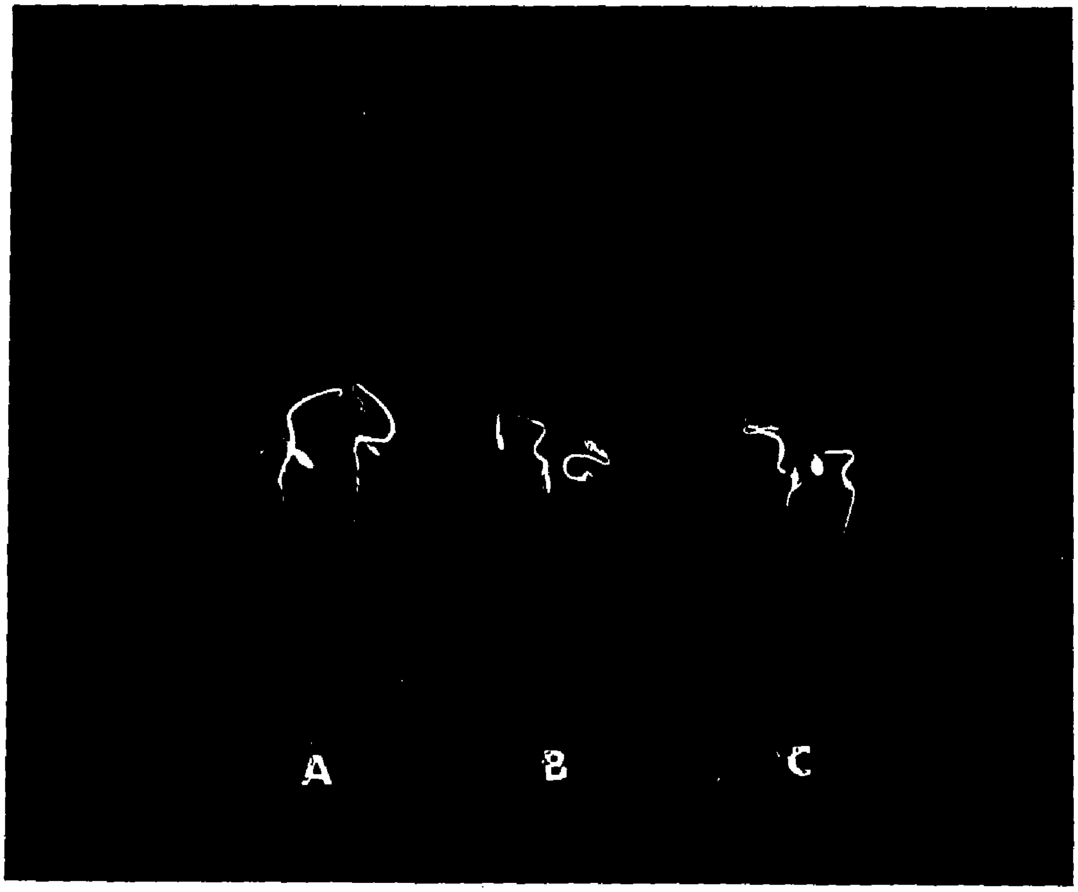
Appendix K

Effect of "plant extract" on cucumber radicle bioassay.

A = Water

B = GA_3

C = Extract



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